PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] : A61K 38/00	A2	(11) International Publication Number: **WO 99/65506** (43) International Publication Date: 23 December 1999 (23.12.99)

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FRIEDLAND, Hillel,
David [DE/CA]; 139 East 26th Avenue, Vancouver,
British Columbia V5V 2G8 (CA). KRIEGER, Timothy, J.
[CA/CA]; 9557 Francis Road, Richmond, British Columbia
V6Y 3A6 (CA). TAYLOR, Robert [CA/CA]; 12683 15A
Avenue, Surrey, British Columbia V4A 1L8 (CA). ERFLE,
Douglas [CA/CA]; 1308 West 7th Avenue, Vancouver,
British Columbia V6H 3W5 (CA). FRASER, Janet,
R. [GB/CA]; #12–1038 West 7th Avenue, Vancouver,
British Columbia V6H 1B3 (CA). WEST, Michael, H., P.
[CA/CA]; 2554 East 19th, Vancouver, British Columbia
V5M 2S3 (CA).

(74) Agent: DEETH WILLIAM WALL; National Bank Building,
Suite 400, 150 York Street, Toronto, Ontario M5H 3S5
(CA).

(54) Title: CANCER THERAPY WITH CATIONIC PEPTIDES

(57) Abstract

Compositions and methods for modifying thera-
peutics with activated polyoxyalkylenes are provided.
Therapeutics that may be modified contain or are
derivatized to contain an amino or nucleophilic group.
Therapeutics include peptides, proteins, nucleic acids,
and the like. In addition, cationic peptides, modified or
unmodified, can be used to treat tumors, either alone
or in combination with a conventional antineoplastic
agent.

A

B

C

CANCER THERAPY WITH CATIONIC PEPTIDES

TECHNICAL FIELD

       The present invention relates generally to polymer-modified
5 therapeutics, such as peptides, nucleic acids, antibiotics, and their uses.

BACKGROUND OF THE INVENTION

       Before the 1940's, the principal nonsurgical approaches to tumor
treatment required the use of X-rays or radium therapy (see, for example, Barrows,
10 "Antineoplastic and Immunoactive Drugs," in *Remington: The Science and Practice of
Pharmacy, 19th Edition*, Gennaro (Ed.), pages 1236-1262 (Mack Publishing Co.
1995)). Although there has been marked progress in the development of antineoplastic
agents, the design of effective pharmaceuticals still faces numerous challenges. One
difficulty is that the main difference between mature normal tissues and tumors is that
15 the cell proliferation rate in most normal tissues equals the rate of cell death, while cell
proliferation exceeds the death rate in tumor tissues. Consequently, an agent that is
toxic for tumor cells may well induce toxicity in normal cells.

       In addition to the problem of selectivity, tumors can become progressively
unresponsive to a particular antineoplastic agent, or even to a combination of various
20 agents. This phenomenon of acquired drug resistance is believed to be due to the
selection and growth of drug-resistant mutant tumor cells from an initial cell population
(see, for example, Deuchars *et al., Sem. Oncol. 16*:156, 1989). Studies have shown that,
under certain selection conditions, cells may acquire simultaneous resistance to a diverse
group of drugs that are unrelated to the selecting agent in structure, cellular target and
25 mode of action (see, for example, Bradley *et al., Biochim. Biophys. Acta 948*:87, 1988;
Deuchars *et al., Sem. Oncol. 16*:156, 1989). Many of the drugs affected by this
"multidrug-resistance" (MDR) phenotype are important in current treatment protocols,
such as vincristine, actinomycin D, and adriamycin.

       In animal cells, the MDR phenotype is associated with over-expression of
30 a 170 kilodalton membrane glycoprotein, designated "gp170" or "P-glycoprotein." P-
glycoprotein is a transmembrane protein responsible for an ATP-dependent efflux of a
broad spectrum of structurally and functionally distinct drugs from multidrug-resistant
cells (Riordan *et al., Pharmacol. Ther. 28*:51, 1985; Endicott *et al., Ann. Rev. Biochem.
58*:137, 1989; Kane *et al., J. Bioenerg. Biomembr. 22*:593, 1990; Efferth *et al., Urol. Res.
35 18*:309, 1990).

Considerable effort has been employed to overcome the multidrug-resistant phenotype. Typical strategies have required pharmacological agents that enhance the intracellular accumulation of the cancer drugs by inhibiting the multidrug transporter (see, for example, Ford *et al., Pharmacol. Rev. 42*:155, 1990). In early

5 clinical trials, however, multidrug-resistant reversing drugs have shown major side effects unrelated to the inhibition of P-glycoprotein, such as cardiac toxicity (verapamil) or immunosuppression (cyclosporin A), which limit the dosage of drug that can be administered (see, for example, Ozols *et al., J. Clin. Oncol. 5*:641, 1987; Dalton *et al., J. Clin. Oncol. 7*:415, 1989; Cano-Gauci *et al., Biochem. Pharmacol. 36*: 2115, 1987).

10 Thus, there has been limited success in reversing MDR *in vivo* due to the toxicity of many of these modulators.

Thus, a need exists for antineoplastic agents that have enhanced selectivity for tumor cells and that are effective against tumor cells exhibiting the MDR phenotype.

15 SUMMARY OF THE INVENTION

The present invention provides pharmaceutical compositions that can be used to treat an infection or a tumor. Such compositions comprise at least one cationic peptide and a pharmaceutically acceptable carrier. Illustrative cationic peptides include APO-modified cationic peptides, or peptides such as MBI 11A9CN (I L R W P W W P

20 W W P W R R K), MBI 11A10CN (W W R W P W W P W R R K), MBI 11B7CN (I L R W P W W P W R R K), MBI 11B19CN (I L R W P W R R W P W R R K), MBI 11B20CN (I L R W P W W P W R R K I L M R W P W W P W R R K M A A), MBI 11D19CN (C L R W P W W P W R R K), MBI 11E3CN (i L K K W P W W P W R R k), MBI 11F4CN (I L R W V W W V W R R K), MBI 11F5CN (I L R R W V W W V

25 W R R K), MBI 11F6CN (I L R W W V W W V W W R R K), MBI 11G25CN (L R W W W P W R R K), MBI 11G26CN (L R W P W W P W), MBI 11G28CN (R W W W P W R R K), MBI 11J01CN (R R I W K P K W R L P K R), MBI 11J02CN (W R W W K P K W R W P K W), or MBI 29 (K W K S F I K K L T T A V K K V L T T G L P A L I S).

30 The pharmaceutical compositions may further comprise at least one antineoplastic drug. Suitable antineoplastic drugs include nitrogen mustard, alkyl sulfonate, nitrosourea, triazene, folic acid analog, pyrimidine analog, purine analog, epipodophyllotoxin, and platinum coordination complex.

The present invention also provides methods for treating a subject

35 having a tumor, comprising the step of administering a pharmaceutical composition that comprises at least one cationic peptide and a pharmaceutically acceptable carrier. Illustrative tumors include lymphoma, leukemia, multiple myeloma, breast tumor, lung tumor, ovarian tumor, cervical tumor, uterine tumor, skin tumor, prostate tumor, liver

tumor, and colon tumor. These methods can also be used to treat tumors comprising multidrug resistant cells.

The present invention further contemplates methods for treating a subject having a tumor by administering a pharmaceutical composition comprising at

5   least one cationic peptide, and by administering an antineoplastic agent, such as nitrogen mustard, alkyl sulfonate, nitrosourea, triazene, folic acid analog, pyrimidine analog, purine analog, epipodophyllotoxin, and platinum coordination complex. The antineoplastic agent can be administered prior to the administration of the cationic peptide, concomitantly with the administration of the cationic peptide, or after

10 administration of the cationic peptide.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are identified below and are incorporated by reference in their entirety.

15

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1C present RP-HPLC traces analyzing samples for APO-peptide formation after treatment of activated polysorbate with a reducing agent. APO-MBI-11CN peptides are formed via lyophilization in 200 mM acetic acid-NaOH, pH

20 4.6, 1 mg/ml MBI 11CN, and 0.5% activated polysorbate 80. The stock solution of activated 2.0% polysorbate is treated with (a) no reducing agent, (b) 150 mM 2-mercaptoethanol, or (c) 150 mM sodium borohydride for 1 hour immediately before use.

Figures 2A and 2B present RP-HPLC traces monitoring the formation of

25 APO-MBI 11CN over time in aqueous solution. The reaction occurs in 200 mM sodium carbonate buffer pH 10.0, 1 mg/ml MBI 11CN, 0.5% activated polysorbate 80. Aliquots are removed from the reaction vessel at the indicated time points and immediately analyzed by RP-HPLC.

Figure 3 presents time kill assay results for MBI 11CN, MBI 11F4CN

30 and MBI 11B7CN. The number of colony forming units x 10^{-4} is plotted versus time.

Figure 4 presents CD spectra of MBI 11CN and MBI 11B7CN.

Figure 5 presents graphs showing the activity of MBI 11B7CN against mid-log cells grown in terrific broth (TB) or Luria-Bretani broth (LB).

Figure 6 is a graph presenting the *in vitro* amount of free MBI 11CN in

35 plasma over time. Data is shown for peptide in formulation C1 and formulation D.

Figure 7 is a graph presenting change in *in vivo* MBI 11CN levels in blood at various times after intravenous injection.

Figure 8 is a graph presenting change in *in vivo* MBI 11CN levels in plasma at various times after intraperitoneal injection.

Figure 9 is a graph showing the number of animals surviving an MSSA infection after intraperitoneal injection of MBI 10CN, ampicillin, or vehicle.

5   Figure 10 is a graph showing the number of animals surviving an MSSA infection after intraperitoneal injection of MBI 11CN, ampicillin, or vehicle.

Figure 11 is a graph showing the results of *in vivo* testing of MBI-11A1CN against *S. aureus* (Smith). Formulated peptide at various concentrations is administered by ip injection one hour after infection with *S. aureus* (Smith) by ip

10   injection.

Figure 12 is a graph showing the results of *in vivo* testing of MBI-11E3CN against *S. aureus* (Smith). Formulated peptide at various concentrations is administered by ip injection one hour after infection with *S. aureus* (Smith) by ip injection.

15   Figure 13 is a graph showing the results of *in vivo* testing of: MBI-11F3CN against *S. aureus* (Smith). Formulated peptide at various concentrations is administered by ip injection one hour after infection with *S. aureus* (Smith) by ip injection.

Figure 14 is a graph showing the results of *in vivo* testing of MBI-

20   11G2CN against *S. aureus* (Smith). Formulated peptide at various concentrations is administered by ip injection one hour after infection with *S. aureus* (Smith) by ip injection.

Figure 15 is a graph showing the results of *in vivo* testing of MBI-11CN against *S. aureus* (Smith). Formulated peptide at various concentrations is administered

25   by ip injection one hour after infection with *S. aureus* (Smith) by ip injection.

Figure 16 is a graph showing the results of *in vivo* testing of MBI-11B1CN against *S. aureus* (Smith). Formulated peptide at various concentrations is administered by ip injection one hour after infection with *S. aureus* (Smith) by ip injection.

30   Figure 17 is a graph showing the results of *in vivo* testing of MBI-11B7CN against *S. aureus* (Smith). Formulated peptide at various concentrations is administered by ip injection one hour after infection with *S. aureus* (Smith) by ip injection.

Figure 18 is a graph showing the results of *in vivo* testing of MBI-

35   11B8CN against *S. aureus* (Smith). Formulated peptide at various concentrations is administered by ip injection one hour after infection with *S. aureus* (Smith) by ip injection.

Figure 19 is a graph showing the results of *in vivo* testing of MBI-11G4CN against *S. aureus* (Smith). Formulated peptide at various concentrations is administered by ip injection one hour after infection with *S. aureus* (Smith) by ip injection.

Figures 20A and B display a graph showing the number of animals surviving an *S. epidermidis* infection after intravenous injection of MBI 10CN, gentamicin, or vehicle. Panel A, i.v. injection 15 min post-infection; panel B, i.v. injection 60 min post-infection.

Figure 21 is a graph showing the number of animals surviving an MRSA infection mice after intravenous injection of MBI 11CN, gentamicin, or vehicle.

Figure 22 is a graph showing cytotoxicity of peptides against peripheral blood lymphocytes (PBL), human lymphoma (DOHH-2) and mouse ascites cells P388. The bars indicate the average concentration of peptide killing 50% of the cells (LC_{50}) for replicate assays. 11A3CN, H-ILKKYPYYPYRRK-CN.

Figure 23 is a graph showing cytoxicity of chemotherapeutic agents, doxorubicin and vincristine sulphate towards normal cells, such as PBL and HUVEC, human tumor cell lines (MCF-7, H460, K562, LS180), and mouse P388 tumor cells. The bars indicate the concentration of agent yielding 50% cell survival. A: PBL; B: HUVEC; C: H460; D: K562; E: LS180; F: MCF-7; G: MCF-7-ADR; H: P388; I: P388-ADR.

Figure 24 is a graph showing the cytotoxicity of 11CN and 11CN-T. The bars show the average concentration of peptide killing 50% of the cells (LC_{50}) for replicate assays. Peptide 11CN-T (also designated 11CN-Tw80) is an APO-modified cationic peptide, prepared with TWEEN 80, as described herein. PBL, normal human peripheral blood lymphocytes; HUVEC, normal human umbilical vein endothelial cells; H460, human non-small lung tumor; MCF-7 human breast carcinoma; K562, human erythroleukemia DOHH-2, human B-cell lymphoma, and P388, mouse ascites leukemia.

Figure 25 is a graph showing the cytotoxicity of 11E3CN and 11E3CN-T. The bars show the average concentration of peptide killing 50% of the cells (LC_{50}) for replicate assays. Peptide 11E3CN-T (also designated 11E3CN-Tw80) is an APO-modified cationic peptide, prepared with TWEEN 80, as described herein. PBL, normal human peripheral blood lymphocytes; HUVEC, normal human umbilical vein endothelial cells; H460, human non-small lung tumor; MCF-7 human breast carcinoma; K562, human erythroleukemia DOHH-2, human B-cell lymphoma, and P388, mouse ascites leukemia.

Figure 26 is a graph showing the cytotoxic activity of peptides on MCF-7 wild-type cells and MCF-7 ADR drug-resistant cells. The bars show the average

concentration of peptide killing 50% of the cells (LC$_{50}$) for replicate assays. MBI 28, H-KWKL FKKI GIGA VLKV LTTG LPAL KLTK-OH; MBI 29, H-KWKS FIKK LTTA VKKV LTTG LPALIS-OH. Peptides 11A3-T and MBI29-T (also designated 11A3-Tw80 and MBI29-Tw80, respectively) are APO-modified cationic peptides, prepared with TWEEN 80, as described herein.

Figure 27 is a graph showing the cytotoxic activity of peptides on P388 wild-type cells and P388 ADR drug-resistant cells. The bars show the average concentration of peptide killing 50% of the cells (LC$_{50}$) for replicate assays.

Figure 28 is a graph showing the cytotoxic activity of 11CN on three cell lines, as determined by a MTT assay (IC$_{50}$) and an LDH assay (LC$_{50}$).

Figure 29 is a graph showing the combined cytotoxicity of 11E3CN and doxorubicin on P388 ADR cells. The line with squares (P388 ADR) represents the combination of peptide and doxorubicin at which the IC$_{50}$ was reached, whereas the line without symbols (P388 predicted) represents the combination predicted to yield 50% viability in the absence of synergy.

Figure 30 is a graph showing the combined toxicity of 11CN and doxorubicin against MCF-7 ADR cells. The line with open squares (IC$_{50}$ MCF-7 ADR) represents the concentrations of each agent, 11CN and doxorubicin at which the IC$_{50}$ was reached. The line without symbols (MCF-7ADR predicted) represents the combination of peptide and doxorubicin at which would be predicted to yield 50% viability in the absence of synergy.

DETAILED DESCRIPTION OF THE INVENTION

1.   DEFINITIONS

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

The amino acid designations herein are set forth as either the standard one-letter or three-letter code. A capital letter indicates an L-form amino acid, while a small letter indicates a D-form amino acid.

An "isolated polypeptide" is a polypeptide that is essentially free from contaminating cellular components, such as carbohydrate, lipid, or other proteinaceous impurities associated with the polypeptide in nature.   That a particular protein preparation contains an isolated polypeptide can be shown by the appearance of a single band following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the protein preparation and Coomassie Brilliant Blue staining of the gel.

As used herein, "cationic peptide" refers to a peptide that has a net positive charge within the pH range of 4 - 10. A cationic peptide is at least five amino

acids in length, and has at least one basic amino acid (*e.g.*, arginine, lysine, histidine). Cationic peptides commonly do not have more than 25, 27, 30, 35, or 40 amino acids, and typically contain 12 to 30 amino acid residues.

As used herein, "nucleic acid" or "nucleic acid molecule" refers to any of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acids can be composed of monomers that are naturally-occurring nucleotides (such as deoxyribonucleotides and ribonucleotides), or analogs of naturally-occurring nucleotides (*e.g.*, α-enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have modifications in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. The term "nucleic acid" also includes so-called "peptide nucleic acids," which comprise naturally-occurring or modified nucleic acid bases attached to a polyamide backbone. Nucleic acids can be either single stranded or double stranded.

As used herein, an "antibiotic agent" refers to a molecule that tends to prevent, inhibit, or destroy life. The term "antimicrobial agent" refers to an antibiotic agent specifically directed to a microorganism, while the term "antineoplastic agent" refers to an agent that is toxic to a tumor cell.

2.   CATIONIC PEPTIDES

The present invention contemplates the use of any "cationic peptide," as that term is defined above. For example, suitable cationic peptides include cecropins, normally made by lepidoptera (Steiner et al., *Nature 292*:246, 1981) and diptera (Merrifield et al., *Ciba Found. Symp. 186*:5, 1994), by porcine intestine (Lee et al., *Proc. Nat'l Acad. Sci. USA 86*:9159, 1989), by blood cells of a marine protochordate (Zhao et al., *FEBS Lett. 412*:144, 1997), synthetic analogs of cecropin A, melittin, and cecropin-melittin chimeric peptides (Wade et al., *Int. J. Pept. Protein Res. 40*:429,

8

1992), cecropin B analogs (Jaynes et al., *Plant Sci. 89*:43, 1993), chimeric cecropin A/B hybrids (Düring, *Mol. Breed. 2*:297, 1996), magainins (Zasloff, *Proc. Nat'l Acad. Sci USA 84*:5449, 1987), cathelin-associated antimicrobial peptides from leukocytes of humans, cattle, pigs, mice, rabbits, and sheep (Zanetti et al., *FEBS Lett. 374*:1, 1995),

5 vertebrate α-defensins, such as human neutrophil defensins [HNP 1-4], paneth cell defensins of mouse and human small intestine (Oulette and Selsted, *FASEB J. 10*:1280, 1996; Porter et al., *Infect. Immun. 65*:2396, 1997), vertebrate β-defensins, such as HBD-1 of human epithelial cells (Zhao et al., *FEBS Lett. 368*:331, 1995), HBD-2 of inflamed human skin (Harder et al., *Nature 387*:861, 1997), bovine β-defensins

10 (Russell et al., *Infect. Immun. 64*:1565, 1996), plant defensins, such as Rs-AFP1 of radish seeds (Fehlbaum et al., *J. Biol. Chem. 269*:33159, 1994), α- and β-thionins (Stuart et al., *Cereal Chem. 19*:288, 1942; Bohlmann and Apel, *Annu. Rev. Physiol. Plant Mol. Biol. 42*:227, 1991), γ-thionins (Broekaert et al., *Plant Physiol. 108*:1353, 1995), the anti-fungal drosomycin (Fehlbaum et al., *J. Biol. Chem. 269*:33159, 1994),

15 apidaecins, produced by honey bee, bumble bee, cicada killer, hornet, yellow jacket, and wasp (Casteels et al., *J. Biol. Chem. 269*:26107, 1994; Levashina et al., *Eur. J. Biochem. 233*:694, 1995), cathelicidins, such as indolicidin from bovine neutrophils (Falla et al., *J. Biol. Chem. 277*:19298, 1996), bacteriocins, such as nisin (Delves-Broughton et al., *Antonie van Leeuwenhoek J. Microbiol. 69*:193, 1996), and the

20 protegrins and tachyplesins, which have antifungal, antibacterial and antiviral activities (Tamamura et al., *Biochim. Biophys. Acta 1163*:209, 1993; Aumelas et al., *Eur. J. Biochem. 237*:575, 1996; Iwanga et al., *Ciba Found. Symp. 186*:160, 1994). Illustrative cationic peptides are listed in Table 1.

25

Table 1

Illustrative Cationic Peptides

Group Name	Peptide	Sequence	Reference*
Abaecins	Abaecin	YVPLPNVPQPGRRPFPTFPGQGP FNPKIKWPQGY	Casteels et al. (1990)
Andropins	Andropin	VFIDILDKVENAIHNAAQVGIGF AKPFEKLINPK	Samakovlis et al. (1991)
Apidaecins	Apidaecin IA	GNNRPVYIPQPRPPHPRI	Casteels et al. (1989)
	Apidaecin IB	GNNRPVYIPQPRPPHPRL	Casteels et al. (1989)
	Apidaecin II	GNNRPIYIPQPRPPHPRL	Casteels et al. (1989)
AS	AS-48	7.4 kDa	Galvez et al. (1989)
Bactenecins	Bactenecin	RLCRIVVIRVCR	Romeo et al. (1988)

Group Name	Peptide	Sequence	Reference*
Bac	Bac5	RFRPPIRRPPIRPPFYPPFRPPIRPP IFPPIRPPFRPPLRFP	Frank et al. (1990)
	Bac7	RRIRPRPPRLPRPRPRPLPFPRPG PRPIPRPLPFPRPGPRPIPRPLPFP RPGPRPIPRP	Frank et al. (1990)
Bactericidins	Bactericidin B2	WNPFKELERAGQRVRDAVISAA PAVATVGQAAAIARG*	Dickinson et al. (1988)
	Bactericidin B-3	WNPFKELERAGQRVRDAIISAG PAVATVGQAAAIARG	Dickinson et al. (1988)
	Bactericidin B-4	WNPFKELERAGQRVRDAIISAA PAVATVGQAAAIARG*	Dickinson et al. (1988)
	Bactericidin B-5P	WNPFKELERAGQRVRDAVISAA AVATVGQAAAIARGG*	Dickinson et al. (1988)
Bacteriocins	Bacteriocin C3603	4.8 kDa	Takada et al. (1984)
	Bacteriocin IY52	5 kDa	Nakamura et al. (1983)
Bombinins	Bombinin	GIGALSAKGALKGLAKGLAZHF AN*	Csordas and Michl (1970)
	BLP-1	GIGASILSAGKSALKGLAKGLAE HFAN*	Gibson et al. (1991)
	BLP-2	GIGSAILSAGKSALKGLAKGLAE HFAN*	Gibson et al. (1991)
Bombolitins	Bombolitin BI	IKITTMLAKLGKVLAHV*	Argiolas and Pisano (1985)
	Bombolitin BII	SKITDILAKLGKVLAHV*	Argiolas and Pisano (1985)
BPTI	Bovine Pancreatic Trypsin Inhibitor (BPTI)	RPDFCLEPPYTGPCKARIIRYFY NAKAGLCQTFVYGGCRAKRNN FKSAEDCMRTCGGA	Creighton and Charles (1987)
Brevinins	Brevinin-1E	FLPLLAGLAANFLPKIFCKITRK C	Simmaco et al. (1993)
	Brevinin-2E	GIMDTLKNLAKTAGKGALQSLL NKASCKLSGQC	Simmaco et al. (1993)
Cecropins	Cecropin A	KWKLFKKIEKVGQNIRDGIIKA GPAVAVVGQATQIAK*	Gudmundsson et al. (1991)

Group Name	Peptide	Sequence	Reference*
	Cecropin B	KWKVFKKIEKMGRNIRNGIVKA GPAIAVLGEAKAL*	Xanthopoulos et al. (1988)
	Cecropin C	GWLKKLGKRIERIGQHTRDATI QGLGIAQQAANVAATARG*	Tryselius et al. (1992)
	Cecropin D	WNPFKELEKVGQRVRDAVISAG PAVATVAQATALAK*	Hultmark et al. (1982)
	Cecropin P₁	SWLSKTAKKLENSAKKRISEGIA IAIQGGPR	Lee et al. (1989)
Charybdtoxins	Charybdtoxin	ZFTNVSCTTSKECWSVCQRLHN TSRGKCMNKKCRCYS	Schweitz et al. (1989)
Coleoptericins	Coleoptericin	8.1 kDa	Bulet et al. (1991)
Crabolins	Crabolin	FLPLILRKIVTAL*	Argiolas and Pisano (1984)
α-Defensins	Cryptdin 1	LRDLVCYCRSRGCKGRERMNG TCRKGHLLYTLCCR	Selsted et al. (1992)
	Cryptdin 2	LRDLVCYCRTRGCKRRERMNG TCRKGHLMYTLCCR	Selsted et al. (1992)
	MCP1	VVCACRRALCLPRERRAGFCRI RGRIHPLCCRR	Selsted et al. (1983)
	MCP2	VVCACRRALCLPLERRAGFCRI RGRIHPLCCRR	Ganz et al. (1989)
	GNCP-1	RRCICTTRTCRFPYRRLGTCIFQ NRVYTFCC	Yamashita and Saito (1989)
	GNCP-2	RRCICTTRTCRFPYRRLGTCLFQ NRVYTFCC	Yamashita and Saito (1989)
	HNP-1	ACYCRIPACIAGERRYGTCIYQG RLWAFCC	Lehrer et al. (1991)
	HNP-2	CYCRIPACIAGERRYGTCIYQGR LWAFCC	Lehrer et al. (1991)
	NP-1	VVCACRRALCLPRERRAGFCRI RGRIHPLCCRR	Ganz et al. (1989)
	NP-2	VVCACRRALCLPLERRAGFCRI RGRIHPLCCRR	Ganz et al. (1989)
	RatNP-1	VTCYCRRTRCGFRERLSGACGY RGRIYRLCCR	Eisenhauer et al. (1989)

Group Name	Peptide	Sequence	Reference*
	RatNP-2	VTCYCRSTRCGFRERLSGACGY RGRIYRLCCR	Eisenhauer et al. (1989)
β-Defensins	BNBD-1	DFASCHTNGGICLPNRCPGHMI QIGICFRPRVKCCRSW	Selsted et at. (1993)
	BNBD-2	VRNHVTCRINRGFCVPIRCPGRT RQIGTCFGPRIKCCRSW	Selsted et al. (1993)
	TAP	NPVSCVRNKGICVPIRCPGSMK QIGTCVGRAVKCCRKK	Diamond et al. (1991)
Defensins-insect	Sapecin	ATCDLLSGTGINHSACAAHCLL RGNRGGYCNGKAVCVCRN	Hanzawa et al. (1990)
	Insect defensin	GFGCPLDQMQCHRHCQTITGRS GGYCSGPLKLTCTCYR	Bulet et al. (1992)
Defensins-scorpion	Scorpion defensin	GFGCPLNQGACHRHCRSIRRRG GYCAGFFKQTCTCYRN	Cociancich et al. (1993)
Dermaseptins	Dermaseptin	ALWKTMLKKLGTMALHAGKA ALGAADTISQTQ	Mor et al. (1991)
Diptericins	Diptericin	9 kDa	Reichhardt et al. (1989)
Drosocins	Drosocin	GKPRPYSPRPTSHPRPIRV	Bulet et al. (1993)
Esculentins	Esculentin	GIFSKLGRKKIKNLLISGLKNVG KEVGMDVVRTGIDIAGCKIKGE C	Simmaco et al. (1993)
Indolicidins	Indolicidin	ILPWKWPWWPWRR*	Selsted et al. (1992)
Lactoferricins	Lactoferricin B	FKCRRWQWRMKKLGAPSITCV RRAF	Bellamy et al. (1992b)
Lantibiotics	Nisin	ITSISLCTPGCKTGALMGCNMK TATCHCSIHVSK	Hurst (1981)
	Pep 5	TAGPAIRASVKQCQKTLKATRL FTVSCKGKNGCK	Keletta et al. (1989)
	Subtilin	MSKFDDFDLDVVKVSKQDSKIT PQWKSESLCTPGCVTGALQTCF LQTLTCNCKISK	Banerjee and Hansen (1988)
Leukocins	Leukocin A-val 187	KYYGNGVHCTKSGCSVNWGEA FSAGVHRLANGGNGFW	Hastings et al. (1991)
Magainins	Magainin 1	GIGKFLHSAGKFGKAFVGEIMK S*	Zasloff (1987)

12

Group Name	Peptide	Sequence	Reference*
	Magainin II	GIGKFLHSAKKFGKAFVGEIMNS*	Zasloff (1987)
	PGLa	GMASKAGAIAGKIAKVALKAL*	Kuchler et al. (1989)
	PGQ	GVLSNVIGYLKKLGTGALNAVLKQ	Moore et al. (1989)
	XPF	GWASKIGQTLGKIAKVGLKELIQPK	Sures and Crippa (1984)
Mastoparans	Mastoparan	INLKALAALAKKIL*	Bernheimer and Rudy (1986)
Melittins	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	Tosteson and Tosteson (1984)
Phormicins	Phormicin A	ATCDLLSGTGINHSACAAHCLLRGNRGGYCNGKGVCVCRN	Lambert et al. (1989)
	Phormicin B	ATCDLLSGTGINHSACAAHCLLRGNRGGYCNRKGVCVRN	Lambert et al. (1989)
Polyphemusins	Polyphemusin I	RRWCFRVCYRGFCYRKCR*	Miyata et al. (1989)
	Polyphemusin II	RRWCFRVCYKGFCYRKCR*	Miyata et al. (1989)
Protegrins	Protegrin I	RGGRLCYCRRRFCVCVGR	Kokryakov et al. (1993)
	Protegrin II	RGGRLCYCRRRFCICV	Kikryakov et al. (1993)
	Protegrin III	RGGGLCYCRRRFCVCVGR	Kokryakov et al. (1993)
Royalisins	Royalisin	VTCDLLSFKGQVNDSACAANCLSLGKAGGHCEKGVCICRKTSFKDLWDKYF	Fujiwara et al. (1990)
Sarcotoxins	Sarcotoxin IA	GWLKKIGKKIERVGQHTRDATIQGLGIAQQAANVAATAR*	Okada and Natori (1985b)
	Sarcotoxin IB	GWLKKIGKKIERVGQHTRDATIQVIGVAQQAANVAATAR*	Okada and Natori (1985b)
Seminal plasmins	Seminalplasmin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV	Reddy and Bhargava (1979)
Tachyplesins	Tachyplesin I	KWCFRVCYRGICYRRCR*	Nakamura et al. (1988)
	Tachyplesin II	RWCFRVCYRGICYRKCR*	Muta et al. (1990)
Thionins	Thionin BTH6	KSCCKDTLARNCYNTCRFAGGSRPVCAGACRCKIISGPKCPSDYPK	Bohlmann et al. (1988)

Group Name	Peptide	Sequence	Reference*
Toxins	Toxin 1	GGKPDLRPCIIPPCHYIPRPKPR	Schmidt et al. (1992)
	Toxin 2	VKDGYIVDDVNCTYFCGRNAY CNEECTKLKGESGYCQWASPY GNACYCKLPDHVRTKGPGRCH	Bontems et al. (1991)

*Argiolas and Pisano, *JBC* 259:10106 (1984); Argiolas and Pisano, *JBC* 260:1437 (1985); Banerjee and Hansen, *JBC* 263:9508 (1988); Bellamy et al., *J. Appl. Bacter.* 73:472 (1992); Bernheimer and Rudy, *BBA* 864:123 (1986); Bohlmann et al., *EMBO J.* 7:1559 (1988); Bontems et al., *Science* 254:1521 (1991); Bulet et al., *JBC* 266:24520 (1991); Bulet et al., *Eur. J. Biochem.*

5   209:977 (1992); Bulet et al., *JBC* 268:14893 (1993); Casteels et al., *EMBO J.* 8:2387 (1989); Casteels et al., *Eur. J. Biochem.* 187:381 (1990); Cociancich et al., *BBRC* 194:17 (1993); Creighton and Charles, *J. Mol. Biol.* 194:11 (1987); Csordas and Michl, *Monatsh Chemistry* 101:82 (1970); Diamond et al., *PNAS* 88:3952 (1991); Dickinson et al., *JBC* 263:19424 (1988); Eisenhauer et al., *Infect. and Imm.* 57:2021 (1989); Frank et al., *JBC* 26518871 (1990); Fujiwara et al., *JBC* 265:11333 (1990); Gálvez et al., *Antimicrobial Agents and Chemotherapy* 33:437 (1989); Ganz et al., *J. Immunol.* 143:1358 (1989); Gibson et al., *JBC* 266:23103

10   (1991); Gudmundsson et al., *JBC* 266:11510 (1991); Hanzawa et al., *FEBS Letters* 269:413 (1990); Hastings et al., *J. Bacteriology* 173:7491 (1991); Hultmark et al., *Eur. J. Biochem.* 127:207 (1982); Hurst, *Adv. Appl. Micro.* 27:85 (1981); Kaletta et al., *Archives of Microbiology* 152:16 (1989); Kokryakov et al., *FEBS Letters* 327:231 (1993); Kuchler et al., *Eur. J. Biochem.* 179:281 (1989); Lambert et al., *PNAS* 86:262 (1989); Lee et al., *PNAS* 86:9159 (1989); Lehrer et al., *Cell* 64:229 (1991); Miyata et al., *J. Biochem.* 106:663 (1989); Moore et al., *JBC* 266:19851 (1991); Mor et al., *Biochemistry* 30:8824 (1991); Muta et al., *J.*

15   *Biochem.* 108:261 (1990); Nakamura et al., *JBC* 263:16709 (1988); Nakamura et al., *Infection and Immunity* 39:609 (1983); Okada and Natori, *Biochem. J.* 229:453 (1985); Reddy and Bhargava, *Nature* 279:725 (1979); Reichhart et al., *Eur. J. Biochem.* 182:423 (1989); Romeo et al., *JBC* 263:9573 (1988); Samakovlis et al., *EMBO J.* 10:163 (1991); Schmidt et al., *Toxicon* 30:1027 (1992); Schweitz et al., *Biochem.* 28:9708 (1989); Selsted et al., *JBC* 258:14485 (1983); Selsted et al., *JBC* 267:4292 (1992); Simmaco et al., *FEBS Lett.* 324:159 (1993); Sures and Crippa, *PNAS* 81:380 (1984); Takada et al., *Infect. and Imm.* 44:370

20   (1984); Tosteson and Tosteson, *Biophysical J.* 45:112 (1984); Tryselius et al., *Eur. J. Biochem.* 204:395 (1992); Xanthopoulos et al., *Eur. J. Biochem.* 172:371 (1988); Yamashita and Saito, *Infect. and Imm.* 57:2405 (1989); Zasloff, *PNAS* 84:5449 (1987).

Cationic peptides can be produced using well-established techniques of peptide synthesis. Alternatively, expression vectors can be constructed that comprise a

25   nucleic acid molecule encoding a cationic peptide. Nucleic acid molecules encoding cationic peptides can be isolated from natural sources as described in the references cited above. Cationic peptide genes can also be obtained by automated synthesis of nucleic acid molecules or by using the polymerase chain reaction (PCR) with oligonucleotide primers having nucleotide sequences that are based upon known

30   nucleotide sequences of cationic peptides. In the latter approach, a cationic peptide gene is synthesized using mutually priming long oligonucleotides (see, for example, Ausubel et al. (eds.), *Short Protocols in Molecular Biology*, 3rd Edition, pages 8-8 to 8-9 (John Wiley & Sons 1995) ["Ausubel (1995)"]). Established techniques using the

14

polymerase chain reaction provide the ability to synthesize DNA molecules at least two kilobases in length (Adang et al., *Plant Molec. Biol. 21*:1131, 1993; Bambot et al., *PCR Methods and Applications 2*:266, 1993; Dillon et al., "Use of the Polymerase Chain Reaction for the Rapid Construction of Synthetic Genes," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 263-268, (Humana Press, Inc. 1993); Holowachuk et al., *PCR Methods Appl. 4*:299, 1995).

As noted above, analogs of natural cationic peptides can also be recombinantly produced by the presently described methods. Amino acid sequences of novel cationic peptides are disclosed, for example, by Falla et al., WO 97/08199, and by Fraser et al., WO 98/07745. For example, useful cationic peptides include the following peptides, in which the amino acids are denoted by the one-letter amino acid code and lower case letters represent the D-form of the amino acid:

Apidaecin IA	G N N R P V Y I P Q P R P P H P R I
Deber A2KA2	K K A A A K A A A A A K A A W A A K A A A K K K K
MBI 10	I L P W K W P W W P W R R
MBI 10CN	I L P W K W P W W P W R R
MBI 11	I L K K W P W W P W R R K
MBI 11CN	I L K K W P W W P W R R K
MBI 11CNR	K R R W P W W P W K K L I
MBI 11A1CN	I L K K F P F F P F R R K
MBI 11A2CN	I L K K I P I I P I R R K
MBI 11A3CN	I L K K Y P Y Y P Y R R K
MBI 11A4CN	I L K K W P W P W R R K
MBI 11A5CN	I L K K Y P W Y P W R R K
MBI 11A6CN	I L K K F P W F P W R R K
MBI 11A7CN	I L K K F P F W P W R R K
MBI 11A8CN	I L R Y V Y Y V Y R R K
MBI 11A9CN	I L R W P W W P W W P W R R K
MBI 11A10CN	W W R W P W W P W R R K
MBI 11B1CN	I L R R W P W W P W R R K
MBI 11B2CN	I L R R W P W W P W R K
MBI 11B3CN	I L K W P W W P W R R K
MBI 11B4CN	I L K K W P W W P W R K
MBI 11B5CN	I L K W P W W P W R K
MBI 11B7CN	I L R W P W W P W R R K
MBI 11B7CNR	K R R W P W W P W R L I
MBI 11B8CN	I L W P W W P W R R K

	MBI 11B9CN	I L R R W P W W P W R R R
	MBI 11B10CN	I L K K W P W W P W K K K
	MBI 11B16CN	I L R W P W W P W R R K I M I L K K A G S
	MBI 11B17CN	I L R W P W W P W R R K M·I L K K A G S
5	MBI 11B18CN	I L R W P W W P W R R K D M I L K K A G S
	MBI 11B19CN	I L R W P W R R W P W R R K
	MBI 11B20CN	I L R W P W W P W R R K I L M R W P W W P W R R K M A A
	MBI 11C3CN	I L K K W A W W P W R R K
	MBI 11C4CN	I L K K W P W W A W R R K
10	MBI 11C5CN	W W K K W P W W P W R R K
	MBI 11D1CN	L K K W P W W P W R R K
	MBI 11D3CN	P W W P W R R K
	MBI 11D4CN	I L K K W P W W P W R R K M I L K K A G S
	MBI 11D5CN	I L K K W P W W P W R R M I L K K A G S
15	MBI 11D6CN	I L K K W P W W P W R R I M I L K K A G S
	MBI 11D9M8	W W P W R R K
	MBI 11D10M8	I L K K W P W
	MBI 11D11H	I L K K W P W W P W R R K M
	MBI 11D12H	I L K K W P W W P W R R M
20	MBI 11D13H	I L K K W P W W P W R R I M
	MBI 11D14CN	I L K K W W W P W R K
	MBI 11D15CN	I L K K W P W W W R K
	MBI 11D18CN	W R I W K P K W R L P K W
	MBI 11D19CN	C L R W P W W P W R R K
25	MBI 11E1CN	i L K K W P W W P W R R K
	MBI 11E2CN	I L K K W P W W P W R R k
	MBI 11E3CN	i L K K W P W W P W R R k
	MBI 11F1CN	I L K K W V W W V W R R K
	MBI 11F2CN	I L K K W P W W V W R R K
30	MBI 11F3CN	I L K K W V W W P W R R K
	MBI 11F4CN	I L R W V W W V W R R K
	MBI 11F4CNR	K R R W V W W V W R L I
	MBI 11F5CN	I L R R W V W W V W R R K
	MBI 11F6CN	I L R W W V W W V W W R R K
35	MBI 11G2CN	I K K W P W W P W R R K
	MBI 11G3CN	I L K K P W W P W R R K
	MBI 11G4CN	I L K K W W W P W R R K
	MBI 11G5CN	I L K K W P W W W R R K

16

MBI 11G6CN	I L K K W P W W P R R K
MBI 11G7CN	I L K K W P W W P W R R
MBI 11G13CN	I L K K W P W W P W K
MBI 11G14CN	I L K K W P W W P W R
MBI 11G24CN	L W P W W P W R R K
MBI 11G25CN	L R W W W P W R R K
MBI 11G26CN	L R W P W W P W
MBI 11G27CN	W P W W P W R R K
MBI 11G28CN	R W W W P W R R K
MBI 11H1CN	A L R W P W W P W R R K
MBI 11H2CN	I A R W P W W P W R R K
MBI 11H3CN	I L A W P W W P W R R K
MBI 11H4CN	I L R A P W W P W R R K
MBI 11H5CN	I L R W A W W P W R R K
MBI 11H6CN	I L R W P A W P W R R K
MBI 11H7CN	I L R W P W A P W R R K
MBI 11H8CN	I L R W P W W A W R R K
MBI 11H9CN	I L R W P W W P A R R K
MBI 11H10CN	I L R W P W W P W A R K
MBI 11H11CN	I L R W P W W P W R A K
MBI 11H12CN	I L R W P W W P W R R A
MBI 11J01CN	R R I W K P K W R L P K R
MBI 11J02CN	W R W W K P K W R W P K W
MBI 21A1	K K W W R R V L S G L K T A G P A I Q S V L N K
MBI 21A2	K K W W R R A L Q G L K T A G P A I Q S V L N K
MBI 21A10	K K W W R R V L K G L S S G P A L S N V
MBI 22A1	K K W W R R A L Q A L K N G L P A L I S
MBI 26	K W K S F I K K L T S A A K K V V T T A K P L I S S
MBI 27	K W K L F K K I G I G A V L K V L T T G L P A L I S
MBI 28	K W K L F K K I G I G A V L K V L T T G L P A L K L T K
MBI 29	K W K S F I K K L T T A V K K V L T T G L P A L I S
MBI 29A2	K W K S F I K N L T K V L K K V V T T A L P A L I S
MBI 29A3	K W K S F I K K L T S A A K K V L T T G L P A L I S
MBI 29F1	K W K L F I K K L T P A V K K V L L T G L P A L I S
MBI 31	G K P R P Y S P I P T S P R P I R Y
REWH 53A5	R L A R I V V I R V A R

In the above list, "CN" indicates an amidated C-terminus, an "H" suffix indicates homoserine at the C-terminus, an "M" suffix indicates a MAP branched peptide, and an "R" suffix indicates retro-synthesized peptide.

One type of cationic peptide analog is a peptide that has one or more conservative amino acid substitutions, compared with the amino acid sequence of a naturally occurring cationic peptide. For example, a cationic peptide analog can be devised that contains one or more amino acid substitutions of a known cationic peptide sequence, in which an alkyl amino acid is substituted for an alkyl amino acid in the natural amino acid sequence, an aromatic amino acid is substituted for an aromatic amino acid in the natural amino acid sequence, a sulfur-containing amino acid is substituted for a sulfur-containing amino acid in the natural amino acid sequence, a hydroxy-containing amino acid is substituted for a hydroxy-containing amino acid in the natural amino acid sequence, an acidic amino acid is substituted for an acidic amino acid in the natural amino acid sequence, a basic amino acid is substituted for a basic amino acid in the natural amino acid sequence, or a dibasic monocarboxylic amino acid is substituted for a dibasic monocarboxylic amino acid in the natural amino acid sequence.

Among the common amino acids, for example, a "conservative amino acid substitution" is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) serine and threonine, (4) aspartate and glutamate, (5) glutamine and asparagine, and (6) lysine, arginine and histidine.

Nucleotide sequences encoding such "conservative amino acid" analogs can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like (see Ausubel (1995) at pages 8-10 to 8-22; and McPherson (ed.), *Directed Mutagenesis: A Practical Approach* (IRL Press 1991)).

Although one objective in constructing a cationic peptide variant may be to improve its activity, it may also be desirable to alter the amino acid sequence of a naturally occurring cationic peptide to enhance its production in a recombinant host cell. For example, a nucleotide sequence encoding a radish cationic peptide may include a codon that is commonly found in radish, but is rare for *E. coli*. The presence of a rare codon may have an adverse effect on protein levels when the radish cationic peptide is expressed in recombinant *E. coli*. Methods for altering nucleotide sequences to alleviate this codon usage problem are well known to those of skill in the art (see, for example, Kane, *Curr. Opin. Biotechnol.* *6*:494, 1995, Makrides, *Microbiol. Rev.* *60*:512, 1996, and Brown (Ed.), *Molecular Biology LabFax* (BIOS Scientific Publishers, Ltd. 1991), which provides a codon usage table on pages 245-253).

Yet another type of cationic peptide variant is a cationic peptide which has been conjugated with a bioactive agent, as described below.

Additional cationic peptide variants have one or more amino acids altered to a corresponding D-amino acid. For example, the N-terminal and/or C-terminal amino acid can be a D-amino acid. Certain cationic peptide variants are acetylated at the N-terminal amino acid, and/or amidated (or esterified) at the C-terminal amino acid. Moreover, a cationic peptide variant can be modified by incorporation of homoserine/homoserine lactone at the C-terminal amino acid.

Typically, cationic peptide analogs should exhibit at least 50%, and preferably, greater than 70, 80 or 90%, of the activity of the corresponding naturally occurring cationic peptide. The antibiotic activity of such analogs can be determined using a standard method, such as the assays described herein. As an illustration, Example 12 describes an *in vivo* assay to measure anti-microbial activity. An *in vivo* assay can also be used to evaluate the activity of cationic peptide analogs for treatment of tumors. Alternatively, *in vitro* assays can provide a simple test for antineoplastic analogs, such as the methylthiazoltetrazolium (MTT) and lactate dehydrogenase (LDH) assays. The MTT assay is a tetrazolium dye colormetric assay that measures cell viability, while the LDH assay measures cell cytotoxicity.

As an illustration, tests were performed to evaluate the *in vitro* efficacy of cationic peptides as cytotoxic agents using tumor cell lines and normal cell lines, such as Human Umbilical Vein Endothelial Cells (HUVEC) and peripheral blood lymphocytes (PBL). In the MTT clonogenic assay, cell viability was measured over a three day period. Briefly, the MTT assay was performed by plating cells at concentrations, such that the cells would not reach confluence during the three day incubation period, approximately 1000-3000 cells/well for hematopoietic and solid tumor cells. HUVECs were seeded at 5,000 to 10,000 cells/ml in Falcon T25 flasks and grown to a density of approximately 10^5 cells/ml. Peripheral blood lymphocytes were seeded at 20000 cells/ well. The final volume in the well after addition of peptide was typically 200 µl. Plates were incubated for 72 hours in a humidified atmosphere containing 5% CO_2. After three days incubation, 50 µl of 1 mg/ml MTT were added to each well, and the plate was incubated for 4-8 hours at 37°C. The plate was then developed by removing an aliquot of supernatant from each well and solubilizing the insoluble formazan product with DMSO. Plates were agitated for 10 to 60 seconds and the OD_{570} was determined with a Dynatek microplate reader. The IC_{50} concentration, defined as the concentration of peptide that reduces survival to 50% of untreated cell level, was recorded. The use of the MTT assay is well-known to those of skill in the art (see, for example, Ciapetti et al., *Biomaterials 14*:359, 1993, Hanelt et al.,

Mycopathologia 128:167, 1994, and Sieuwerts et al., *Eur. J. Clin. Chem. Clin. Biochem. 33*:813, 1995).

Cell cytotoxicity against both tumor and normal cells was also determined by release of lactate dehydrogenase into tissue culture supernatants using an
5   assay kit (Promega Corp. USA) LDH cell lysis assay. All cells were greater than 95%. viable by trypan blue exclusion prior to initiation of the assay. To eliminate possible effects from various tissue culture additives, lysis assays were performed in HANKS; balance salt solution pH 7.4. Untreated cells maintained their viability in HANKS; buffer during four hour incubation.. Attachment of growing tumor cells MCF-7 and
10 H460 was maintained in RMPI with 10% heat-inactivated FBS, and the cells were. seeded at 1 X 10^4 cells per well in 96 well microtiter Falcon flat bottom plates, and cultured overnight in a humidified atmosphere at 37°C, 5% CO_2. Prior to addition of peptide, tissue culture medium was removed, wells were rinsed once with HANKS· balanced salt buffer, and then 100 µl Hanks buffer were added to each well.
15 Hematopoietic cells were washed in Hanks buffer saline, and then seeded at 2 X 104 cells/well in microtiter plates in a volume of 100 µl per well. PBL were seeded at. 50,000 to 100,000 cells per well. HUVEC cells were grown to confluence and maintained for three days prior to exposure to peptide. Medium was replaced on alternate days (confluent cultures). For non-confluent HUVEC cultures, cells were
20 assayed one day after seeding.

A two fold dilution series of peptides diluted in HANKS were typically tested in triplicate. Peptides were also diluted in HANKS buffer and added to cells to a final volume of 200 µl. Microtiter plates were incubated for four hours at 37°C, and then centrifuged at 1000 rpm for 5 minutes. The release of LDH as a fraction of the
25 maximum LDH release (100% lysed cells) was determined. LDH activity was measured with a colorometric couple enzyme assay (Promega.). Fifty microliters of supernatant were removed from each well and assayed for LDH activity. The fraction of LDH released was determined in triplicate. The concentration required to cause release of 50% of the maximum LDH release (100% lysed cells) (LC_{50}) was recorded
30 for each peptide.

3.   POLYMER MODIFICATION OF THERAPEUTIC AGENTS

The present invention provides methods and compositions for modifying a compound with a free amine group. The amine group may be part of the native
35 structure of the compound or added by a chemical method. Thus, peptides, proteins, certain antibiotics, nucleic acids and the like can be modified with an activated polyoxyalkylene and derivatives. When the compounds are peptides or proteins, the modified or derivatized forms are referred to herein as "APO-modified peptides" or

20

"APO-modified proteins". Similarly, modified forms of antibiotics are referred to as "APO-modified antibiotics." APO-modified compounds (e.g., APO-cationic peptides) generally exhibit improved pharmacological properties.

5   **A.   *Characteristics of an activated polyoxyalkylene reagent***

As discussed herein, a suitable reagent for formation of APO-modified compounds (e.g., peptides and proteins) comprises a hydrophobic region and a hydrophilic region, and optionally a linker. The hydrophobic region is a lipophilic compound with a suitable functional group for conjugation to the hydrophilic region or

10   linker.   The hydrophilic region is a polyoxyalkylene.   As used herein, "polyoxyalkylene" refers to 2 or 3 carbon polyoxyalkylene polymers. The polymer chain is of a length 2 units or greater. Two carbon polyoxyalkylenes include polyoxyethylene and its derivatives, polyethylene glycol (PEG) of various molecular weights, and its derivatives, such as polysorbate. Three carbon polyoxyalkylenes

15   include polyoxypropylene and derivatives and polypropylene glycol and its derivatives. Derivatives include alkyl- and aryl-polyoxyethylene compounds.

The hydrophobic region is a lipophilic moiety, generally a fatty acid, but may be a fatty alcohol, fatty thiol, hydrocarbons (such as 4-(1,1,3,3-tetramethylbutyl)-cyclohexyl), aryl compounds (such as 4-(1,1,3,3-tetramethylbutyl)-phenyl) and the like,

20   which are also lipophilic compounds. The fatty acid may be saturated or unsaturated. The chain length does not appear to be important, although typically commercially available fatty acids are used and have chain lengths of C_{12-18}. The length may be limited however by solubility or solidity of the compound, that is longer lengths of fatty acids are solid at room temperature. Fatty acids of 12 carbons (lauryl), 14 carbons, 16

25   carbons (palmitate), and 18 carbons (monostearate or oleate) are preferred chain lengths.

The hydrophilic region is a polyoxyalkylene, such as polyethylene, polypropylene glycol monoether (for example Triton X114), and polysorbate. For polysorbate, the ether function is formed by the linkage between the polyoxyethylene

30   chain, preferably having a chain length of from 2 to 100 monomeric units, and the sorbitan group. Polymethylene glycol is unsuitable for administration in animals due to formation of formaldehydes, and glycols with a chain length of ≥ 4 may be insoluble. Mixed polyoxyethylene-polyoxypropylene chains are also suitable.

A linker for bridging the hydrophilic and hydrophobic regions is not

35   required, but if used, should be able to bridge both a polyoxyalkylene and the hydrophobic region. Suitable linkers include sorbitan, sugar alcohols, ethanolamine, ethanolthiol, 2-mercaptoethanol, 1,6 diaminohexane, an amino acid (e.g., glutamine,

lysine), other reduced sugars, and the like. For example, sorbitan forms an ester linkage with the fatty acid in a polysorbate.

Suitable compounds include polyoxyethylenesorbitans, such as the monolaurate, monooleate, monopalmitate, monostearate, trioleate, and tristearate esters. These and other suitable compounds may be synthesized by standard chemical methods or obtained commercially (e.g., Sigma Chemical Co., MO; Aldrich Chemical Co., WI; J.B. Baker,NJ).

B.   Activation of reagent

The reagent is activated by exposure to UV light with free exchange of air or by chemical treatment with ammonium persulfate, or a combination of these methods.

Photoactivation is achieved using a lamp that irradiates at 254 nm or 302 nm. Preferably, the output is centered at 254 nm. Longer wave lengths may require longer activation time. While some evidence exists that fluorescent room light can activate the polysorbates, experiments have shown that use of UV light at 254nm yields maximal activation before room light yields a detectable level of activation.

Air plays an important role in the activation of the polysorbates. Access to air doubles the rate of activation relative to activations performed in sealed containers. A shallow reaction chamber with a large surface area would facilitate oxygen exchange. It is not yet known which gas is responsible; an oxygen derivative is likely, although peroxides are not involved. UV exposure of compounds with ether linkages is known to generate peroxides, which can be detected and quantified using peroxide test strips. In a reaction, hydrogen peroxide at 1 to 10 fold higher level than found in UV-activated material was added to a polysorbate solution in the absence of light. No activation was obtained.

The reagent is placed in a suitable vessel for irradiation. Studies with 2% polysorbate 80 indicate that 254 nm light at 1800 μW/cm2 is completely absorbed by the solution at a depth of 3-4 cm. Thus, the activation rate can be maximized by irradiating a relatively thin layer.

As such, a consideration for the vessel is the ability to achieve uniform irradiation. As noted above, a large shallow reaction chamber is desirable, however, it may be difficult to achieve on a large scale. To compensate, simple stirring that facilitates the replenishment of air in the solution achieves an equivalent result. Thus, if the path length is long or the reaction chamber is not shallow, the reagent may be mixed or agitated. The reagent can be activated in any aqueous solution and buffering is not required.

An exemplary activation takes place in a cuvette with a 1 cm liquid thickness. The reagent is irradiated at a distance of less than 9 cm at 1500 $\mu W/cm^2$ (initial source output) for approximately 24 hours. Under these conditions, the activated reagent converts a minimum of 85% of the peptide to APO-peptide.

5   As noted above, the polyoxyalkylenes can be activated via chemical oxidation with ammonium persulfate. The activation is rapid and the extent of activation increases with the concentration of ammonium persulfate. Ammonium persulfate can be used in a range from about 0.01% -0.5%, and most preferably from 0.025 to 0.1%. If the levels of ammonium persulfate are too high, the peroxide

10   byproducts can have an adverse effect on the compounds being modified. This adverse effect can be diminished by treatment of activated polyoxyalkylenes with mercaptoethanol, or another mild reducing agent, which does not inhibit the formation of APO-therapeutics. Peroxides generated from UV treatment can also be reduced by treatment with mercaptoethanol. Furthermore, as noted above, the UV procedure can

15   be performed in conjunction with chemical activation.

C.   *Modification of peptides or proteins with activated reagent*

The therapeutics are reacted with the APO reagent in either a liquid or solid phase and become modified by the attachment of the APO derivative. The

20   methods described herein for attachment offer the advantage of maintaining the charge on the therapeutic, such as a peptide or protein. When the charge of the peptide is critical to its function, such as the antibiotic activity of cationic peptides described herein, these attachment methods offer additional advantages. Methods that attach groups via acylation result in the loss of positive charge via conversion of amino to

25   amido groups. In addition, no bulky or potentially antigenic linker, such as a triazine group, is known to be introduced by the methods described herein.

As noted above, APO-therapeutic formation occurs in solid phase or in aqueous solution. By way of example, briefly, in the solid phase method, a peptide or other therapeutic is suspended in a suitable buffer, such as an acetate buffer. Other

30   suitable buffers that support APO-therapeutic formation may also be used. The acetate buffer may be sodium, potassium, lithium, and the like. Other acetate solutions, such as HAc or HAc-NaOH, are also suitable. A preferred pH range for the buffer is from 2 to 8.3, although a wider range may be used. When the starting pH of the acetic acid-NaOH buffer is varied, subsequent lyophilization from 200 mM acetic acid buffer

35   yields only the Type I modified peptide (see Example 14). The presence of an alkaline buffer component results in the formation of Type II modified peptides. A typical peptide concentration is 1 mg/ml, which results in 85-95% modified peptide, however other concentrations are suitable. The major consideration for determining

concentration appears to be economic. The activated polymer (APO) is added in molar excess to the therapeutic. Generally, a starting ratio of approximately 2.5:1 (APO:therapeutic) to 5:1 (APO: therapeutic) generates APO-modified therapeutic in good yield.

5   The reaction mix is then frozen (e.g., -80°C) and lyophilized. Sodium acetate disproportionates into acetic acid and NaOH during lyophilization; removal of the volatile acetic acid by the vacuum leaves NaOH dispersed throughout the result solid matrix. This loss of acetic acid is confirmed by a pH increase detected upon dissolution of the lyophilizate. No APO-modified therapeutic is formed in acetate

10   buffer if the samples are only frozen then thawed.

The modification reaction can also take place in aqueous solution. However, APO modifications do not occur at ambient temperature in any acetate buffer system tested regardless of pH. APO modifications also are not formed in phosphate buffers as high as pH 11.5. APO modification does occur in a sodium carbonate buffer

15   at a pH greater than about 8.5. Other buffers may also be used if they support derivitization. A pH range of 9-11 is also suitable, and pH 10 is most commonly used. The reaction occurs in two phases: Type I peptides form first, followed by formation of Type II peptides.

In the present invention, linkage occurs at an amino or a nucleophilic

20   group. The amino group may be a primary amine, a secondary amine, or an aryl amine. Nucleophilic groups that may be APO-modified include, but are not limited to, hydrazine derivatives, hydroxylamine derivatives, and sulfhydryl compounds. Preferably, the modification occurs at an amino group, more preferably at a primary or secondary amino group, and most preferably at a primary amino group. Examples of

25   compounds that have modified by the solid phase method are listed in Table 2.

Table 2

Compound	Action	Modification
Amoxicillin	penicillin antibiotic	Yes
Amphotericin B	anti-fungal	No
Ampicillin	penicillin antibiotic	Yes
Bacitracin	peptide antibiotic	Yes
Cephalosporin C	aminoglycoside antibiotic	No
Ciprofloxacin	quinolone antibiotic	Uncertain*
4,4'-Diaminodiphenyl Sulfone	anti-leprotic	Yes
Gentamycin	aminoglycoside antibiotic	Yes
Gramicidin S	peptide antibiotic	Yes

Sulfadiazine	sulfonamide antibiotic	No
Vancomycin	glycopeptide antibiotic	Yes

*Ciprofloxacin was partially destroyed by the process.

For a peptide, linkage can occur at the α-NH$_2$ of the N-terminal amino acid or ε-NH$_2$ group of lysine. Other primary and secondary amines may also be modified. Complete blocking of all amino groups by acylation (MBI 11CN-Y1) inhibits APO-peptide formation. Thus, modification of arginine or tryptophan residues does not occur. If the only amino group available is the α-amino group (e.g., MBI 11B9CN and MBI 11G14CN), the Type I form is observed. The inclusion of a single lysine (e.g., MBI 11B1CN, MBI 11B7CN, MBI 11B8CN), providing an ε-amino group, results in Type II forms as well. The amount of Type II formed increases for peptides with more lysine residues.

Many antibiotics have free amine groups. Such antibiotics include but are not limited to ampicillin, amoxicillin, amikacin, ciprofloxacin, gentamycin, teicoplanin, tobramycin, and vancomycin. Moreover, in the context of the present invention, other compounds, such as nucleic acids may be APO-modified. To modify nucleic acids, a primary amine group can be added by well known methods. Nucleic acids that may be modified include DNA, RNA, peptide-nucleic acids, and other variants.

Other types of compounds may be modified. For example, viruses lacking an envelope are candidates. Modification may reduce the antigenicity to viral vectors, such as adenoviruses, which are used in gene delivery protocols.

Using the methods described herein, several peptides, including indolicidin, indolicidin analogues, gramicidin and bacitracin-2 have been polymer modified.

D.   Purification and physical properties of APO-modified therapeutics

The APO-modified therapeutics may be purified. In circumstances in which the free therapeutic, such as a peptide is toxic, purification may be necessary to remove unmodified therapeutic and/or unreacted polyoxyalkylenes. Any of a variety of purification methods may be used. Such methods include reversed phase HPLC, precipitation by organic solvent to remove polysorbate, size exclusion chromatography, ion exchange chromatography, filtration and the like. RP-HPLC is preferred. Procedures for these separation methods are well known.

APO-therapeutic formation can result in the generation of products that are more hydrophobic than the parent compound. This property can be exploited to effect separation of the conjugate from free compound by RP-HPLC. As shown herein,

peptide-conjugates are resolved into two populations based on their hydrophobicity as determined by RP-HPLC; the Type I population elutes slightly earlier than the Type II population.

The MBI 11 series of peptides have molecular weights between 1600 and 2500. When run on a Superose 12 column, a size exclusion column, these peptides adsorb to the resin, giving long retention times. In contrast, the APO-modified peptides do not adsorb and elute at 50 kDa (MBI11CN-Tw80) and at 69 kDa (MBI 11A3CN-Tw80), thus demonstrating a large increase in apparent molecular mass (Stokes radius).

An increase in apparent molecular mass could enhance the pharmacokinetics of peptides in particular because increased molecular mass reduces the rate at which peptides and proteins are removed from blood. Micelle formation may offer additional benefits by delivering "packets" of peptide molecules to microorganisms rather than relying on the multiple binding of single peptide molecules. In addition, APO-modified peptides are soluble in methylene chloride or chloroform (e.g., to at least 10 mg/mL), whereas the parent peptide is essentially insoluble. This increased organic solubility may significantly enhance the ability to penetrate tissue barriers and may be exploited for a simplified purification of the APO-peptide. The increased solubility in organic media may also allow the formulation of peptides in oil or lipid based delivery systems which target specific sites, such as solid tumors.

In addition, by circular dichroism (CD) studies, APO-modified peptides are observed to have an altered 3-dimensional conformation. As shown in the Examples, MBI 11CN and MBI 11B7CN have unordered structures in phosphate buffer or 40% aqueous trifluoroethanol (TFE) and form a β-turn conformation only upon insertion into liposomes. In contrast, CD spectra for APO-modified MBI 11CN and APO-modified MBI 11B7CN indicate β-turn structure in phosphate buffer.

Cationic peptides appear to maintain their original charge after modification with an APO, thereby preventing loss of activity sometimes caused by acylation reactions. Moreover, the present methods are not known to introduce antigenic linkers.

E. Biological properties of APO-modified therapeutics

The biological properties of APO-modified therapeutics appear to be improved compared to unmodified therapeutics. For example, modified and unmodified peptides are compared. Because the product consists a peptide of known composition coupled to one or more polyoxyalkylene components derived from a polymeric mixture, defining an exact molecular weight for concentration calculations is not readily achieved. It is possible, however, to determine the concentration by spectrophotometric assay. Such a measurement is used to normalize APO-peptide

concentrations for biological assays. For example, a 1mg/mL MBI11CN-Tw80 solution contains the same amount of cationic peptide as a 1mg/mL solution of the parent peptide, thus allowing direct comparison of toxicity and efficacy data. The modified peptides have an equivalent MIC to unmodified peptides. *In vivo*, however, the modified peptides demonstrate a lower LC50 than the unmodified peptides against a panel of tumor cell lines. Thus, formation of APO-peptides increases the potency of cationic peptides against cancer cells in culture.

In general, the efficacy of a modified therapeutic is determined by *in vitro* and *in vivo* assays used for the unmodified therapeutic. Thus, the assays employed depend upon the therapeutic. Assays for the therapeutics disclosed herein are well known. Assays include those for biological activity, pharmacokinetics, toxicity, adverse reactions, immunogenicity, and the like. Such assays are available to those skilled in the art.

4. ADDITIONAL POLYMER-MODIFIED THERAPEUTIC AGENTS

The present invention provides polymer-modified therapeutics. These therapeutics include any medically relevant compound and need only have a group suitable for modification. As noted herein, many therapeutics have such a group (*e.g.*, amino group). Others can be derivatized to contain a suitable group.

A. *Peptides and proteins*

Within the context of the present invention, any peptide or protein that has an amino group available for modification may be used. Generally, peptides and proteins have an NH2-terminus. In some cases, the N-terminus may be blocked. In such cases, modification can still occur at an ε-amino group of lysine, other nucleophilic group, or the protein (or peptide) can be reacted with a suitable reagent, such as Traut's reagent if a cysteine residue is present, to provide a primary amine for modification.

As used herein, a "peptide" is at least 5 amino acids in length. Unless otherwise indicated, a named amino acid refers to the L-form. Also included within the scope of peptides and proteins are variants that contain amino acid derivatives that have been altered by chemical means, such as methylation (*e.g.*, α methylvaline), amidation, especially of the C-terminal amino acid by an alkylamine (*e.g.*, ethylamine, ethanolamine, and ethylene diamine) and alteration of an amino acid side chain, such as acylation of the ε-amino group of lysine. Other amino acids that may be incorporated include any of the D-amino acids corresponding to the 20 L-amino acids commonly found in proteins, imino amino acids, rare amino acids, such as hydroxylysine, or non-protein amino acids, such as homoserine and ornithine. A peptide or protein may have

none or one or more of these derivatives, and D-amino acids. In addition, a peptide may also be synthesized as a retro-, inverto- or retro-inverto-peptide.

Peptides may be synthesized by standard chemical methods, including synthesis by automated procedure. In general, peptides are synthesized based on the standard solid-phase Fmoc protection strategy with HATU as the coupling agent. The peptide is cleaved from the solid-phase resin with trifluoroacetic acid containing appropriate scavengers, which also deprotects side chain functional groups. Crude peptide is further purified using preparative reversed-phase chromatography. Other purification methods, such as partition chromatography, gel filtration, gel electrophoresis, or ion-exchange chromatography may be used. Other synthesis techniques, known in the art, such as the tBoc protection strategy, or use of different coupling reagents or the like can be employed to produce equivalent peptides.

Peptides may be synthesized as a linear molecule or as branched molecules. Branched peptides typically contain a core peptide that provides a number of attachment points for additional peptides. Lysine is most commonly used for the core peptide because it has one carboxyl functional group and two (alpha and epsilon) amine functional groups. Other diamino acids can also be used. Preferably, either two or three levels of geometrically branched lysines are used; these cores form a tetrameric and octameric core structure, respectively (Tam, *Proc. Natl. Acad. Sci. USA 85*:5409, 1988). To synthesize these multimeric peptides, the solid phase resin is derivatized with the core matrix, and subsequent synthesis and cleavage from the resin follows standard procedures. The multimeric peptide is typically then purified by dialysis against 4 M guanidine hydrochloride then water, using a membrane with a pore size to retain only multimers. The multimeric peptides may be used within the context of this invention as for any of the linear peptides.

Peptides may alternatively be synthesized by recombinant production. Recombinant production is preferred for proteins. A variety of host systems are suitable for production, including bacteria (*e.g.*, *E. coli*), yeast (*e.g.*, *Saccharomyces cerevisiae*), insect (*e.g.*, Sf9), and mammalian cells (*e.g*, CHO, COS-7). Many expression vectors have been developed and are available for each of these hosts. Generally, bacteria cells and vectors that are functional in bacteria are used in this invention. However, at times, it may be preferable to have vectors that are functional in other hosts. Vectors and procedures for cloning and expression in *E. coli* and other organisms are discussed herein and, for example, in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1987) and in Ausubel et al. (*Current Protocols in Molecular Biology*, Greene Publishing Co., 1995).

Peptides and proteins are isolated by standard techniques, such as affinity, size exclusion, or ionic exchange chromatography, HPLC and the like. An isolated peptide or protein should preferably show a major band by Coomassie blue stain of SDS-PAGE that is at least 90% of the material.

B. Antibiotic agents

As used herein, the term "antibiotic agent" includes any molecule that tends to prevent, inhibit or destroy life. As such, agents include anti-bacterial agents, anti-fungicides, anti-viral agents, anti-parasitic agents, and antineoplastic agents. These agents may be isolated from an organism that produces the agent or procured from a commercial source (e.g., pharmaceutical company, such as Eli Lilly, Indianapolis, IN; Sigma, St. Louis, MO). Many of these agents have an amino or nucleophilic group for modification with the disclosed polymers. If no acceptable group is available, the agent may be derivatized by standard chemical methods to incorporated an amino group.

Anti-bacterial antibiotic agents include, but are not limited to, penicillins, cephalosporins, carbacephems, cephamycins, carbapenems, monobactams, aminoglycosides, glycopeptides, quinolones, tetracyclines, macrolides, sulfonamides, and fluoroquinolones (see Table 3). Examples of antibiotics include, but are not limited to, Penicillin G (CAS Registry No.: 61-33-6); Methicillin (CAS Registry No.: 61-32-5); Nafcillin (CAS Registry No.: 147-52-4); Oxacillin (CAS Registry No.: 66-79-5); Cloxacillin (CAS Registry No.: 61-72-3); Dicloxacillin (CAS Registry No.: 3116-76-5); Ampicillin (CAS Registry No.: 69-53-4); Amoxicillin (CAS Registry No.: 26787-78-0); Ticarcillin (CAS Registry No.: 34787-01-4); Carbenicillin (CAS Registry No.: 4697-36-3); Mezlocillin (CAS Registry No.: 51481-65-3); Azlocillin (CAS Registry No.: 37091-66-0); Piperacillin (CAS Registry No.: 61477-96-1); Imipenem (CAS Registry No.: 74431-23-5); Aztreonam (CAS Registry No.: 78110-38-0); Cephalothin (CAS Registry No.: 153-61-7); Cefazolin (CAS Registry No.: 25953-19-9); Cefaclor (CAS Registry No.: 70356-03-5); Cefamandole formate sodium (CAS Registry No.: 42540-40-9); Cefoxitin (CAS Registry No.: 35607-66-0); Cefuroxime (CAS Registry No.: 55268-75-2); Cefonicid (CAS Registry No.: 61270-58-4); Cefmetazole (CAS Registry No.: 56796-20-4); Cefotetan (CAS Registry No.: 69712-56-7); Cefprozil (CAS Registry No.: 92665-29-7); Loracarbef (CAS Registry No.: 121961-22-6); Cefetamet (CAS Registry No.: 65052-63-3); Cefoperazone (CAS Registry No.: 62893-19-0); Cefotaxime (CAS Registry No.: 63527-52-6); Ceftizoxime (CAS Registry No.: 68401-81-0); Ceftriaxone (CAS Registry No.: 73384-59-5); Ceftazidime (CAS Registry No.: 72558-82-8); Cefepime (CAS Registry No.: 88040-23-7); Cefixime (CAS Registry No.: 79350-37-1); Cefpodoxime (CAS Registry No.: 80210-62-4); Cefsulodin (CAS Registry No.: 62587-73-9); Fleroxacin (CAS Registry No.: 79660-72-3);

Nalidixic acid (CAS Registry No.: 389-08-2); Norfloxacin (CAS Registry No.: 70458-96-7); Ciprofloxacin (CAS Registry No.: 85721-33-1); Ofloxacin (CAS Registry No.: 82419-36-1); Enoxacin (CAS Registry No.: 74011-58-8); Lomefloxacin (CAS Registry No.: 98079-51-7); Cinoxacin (CAS Registry No.: 28657-80-9); Doxycycline (CAS Registry No.: 564-25-0); Minocycline (CAS Registry No.: 10118-90-8); Tetracycline (CAS Registry No.: 60-54-8); Amikacin (CAS Registry No.: 37517-28-5); Gentamicin (CAS Registry No.: 1403-66-3); Kanamycin (CAS Registry No.: 8063-07-8); Netilmicin (CAS Registry No.: 56391-56-1); Tobramycin (CAS Registry No.: 32986-56-4); Streptomycin (CAS Registry No.: 57-92-1); Azithromycin (CAS Registry No.: 83905-01-5); Clarithromycin (CAS Registry No.: 81103-11-9); Erythromycin (CAS Registry No.: 114-07-8); Erythromycin estolate (CAS Registry No.: 3521-62-8); Erythromycin ethyl succinate (CAS Registry No.: 41342-53-4); Erythromycin glucoheptonate (CAS Registry No.: 23067-13-2); Erythromycin lactobionate (CAS Registry No.: 3847-29-8); Erythromycin stearate (CAS Registry No.: 643-22-1); Vancomycin (CAS Registry No.: 1404-90-6); Teicoplanin (CAS Registry No.: 61036-64-4); Chloramphenicol (CAS Registry No.: 56-75-7); Clindamycin (CAS Registry No.: 18323-44-9); Trimethoprim (CAS Registry No.: 738-70-5); Sulfamethoxazole (CAS Registry No.: 723-46-6); Nitrofurantoin (CAS Registry No.: 67-20-9); Rifampin (CAS Registry No.: 13292-46-1); Mupirocin (CAS Registry No.: 12650-69-0); Metronidazole (CAS Registry No.: 443-48-1); Cephalexin (CAS Registry No.: 15686-71-2); Roxithromycin (CAS Registry No.: 80214-83-1); Co-amoxiclavuanate; combinations of Piperacillin and Tazobactam; and their various salts, acids, bases, and other derivatives.

Table 3

Class of Antibiotic	Antibiotic	Mode of Action
PENICILLINS		Blocks the formation of new cell walls in bacteria
Natural	Penicillin G, Benzylpenicillin Penicillin V, Phenoxymethylpenicillin	
Penicillinase resistant	Methicillin, Nafcillin, Oxacillin Cloxacillin, Dicloxacillin	
Acylamino-penicillins	Ampicillin, Amoxicillin	
Carboxy-penicillins	Ticarcillin, Carbenicillin	

Ureido-penicillins	Mezlocillin, Azlocillin, Piperacillin	
CARBAPENEMS	Imipenem, Meropenem	Blocks the formation of new cell walls in bacteria
MONOBACTAMS	Aztreonam	Blocks the formation of new cell walls in bacteria
CEPHALOSPORINS 1st Generation 2nd Generation 3rd Generation 4th Generation	 Cephalothin, Cefazolin Cefaclor, Cefamandole Cefuroxime, Cefonicid, Cefmetazole, Cefotetan, Cefprozil Cefetamet, Cefoperazone Cefotaxime, Ceftizoxime Ceftriaxone, Ceftazidime Cefixime,Cefpodoxime, Cefsulodin Cefepime	Prevents formation of new cell walls in bacteria
CARBACEPHEMS **CEPHAMYCINS**	Loracarbef Cefoxitin	Prevents formation of new cell walls in bacteria Prevents formation of new cell walls in bacteria
QUINOLONES	Fleroxacin, Nalidixic Acid Norfloxacin, Ciprofloxacin Ofloxacin, Enoxacin Lomefloxacin, Cinoxacin	Inhibits bacterial DNA synthesis
TETRACYCLINES	Doxycycline, Minocycline, Tetracycline	Inhibits bacterial protein synthesis, binds to 30S

		ribosome subunit.
AMINOGLYCOSIDES	Amikacin, Gentamicin, Kanamycin, Netilmicin, Tobramycin, Streptomycin	Inhibits bacterial protein synthesis, binds to 30S ribosome subunit.
MACROLIDES	Azithromycin, Clarithromycin, Erythromycin	Inhibits bacterial protein synthesis, binds to 50S ribosome subunit
Derivatives of Erythromycin	Erythromycin estolate, Erythromycin stearate Erythromycin ethylsuccinate Erythromycin gluceptate Erythromycin lactobionate	
GLYCOPEPTIDES	Vancomycin, Teicoplanin	Inhibits cell wall synthesis, prevents peptidoglycan elongation.
MISCELLANEOUS	Chloramphenicol	Inhibits bacterial protein synthesis, binds to 50S ribosome subunit.
	Clindamycin	Inhibits bacterial protein synthesis, binds to 50S ribosome subunit.
	Trimethoprim	Inhibits the enzyme dihydrofolate reductase, which activates folic acid.
	Sulfamethoxazole	Acts as antimetabolite of PABA & inhibits synthesis of folic acid
	Nitrofurantoin	Action unknown, but is concentrated in urine where it can act on urinary tract

		bacteria
	Rifampin	Inhibits bacterial RNA polymerase
	Mupirocin	Inhibits bacterial protein synthesis

Anti-fungal agents include, but are not limited to, terbinafine hydrochloride, nystatin, amphotericin B, griseofulvin, ketoconazole, miconazole nitrate, flucytosine, fluconazole, itraconazole, clotrimazole, benzoic acid, salicylic acid, and
5   selenium sulfide.

Anti-viral agents include, but are not limited to, amantadine hydrochloride, rimantadin, acyclovir, famciclovir, foscarnet, ganciclovir sodium, idoxuridine, ribavirin, sorivudine, trifluridine, valacyclovir, vidarabin, didanosine, stavudine, zalcitabine, zidovudine, interferon alpha, and edoxudine.
10   Anti-parasitic agents include, but are not limited to, pirethrins/piperonyl butoxide, permethrin, iodoquinol, metronidazole, diethylcarbamazine citrate, piperazine, pyrantel pamoate, mebendazole, thiabendazole, praziquantel, albendazole, proguanil, quinidine gluconate injection, quinine sulfate, chloroquine phosphate, mefloquine hydrochloride, primaquine phosphate, atovaquone, co-trimoxazole
15   (sulfamethoxazole/trimethoprim), and pentamidine isethionate.

Antineoplastic agents include, but are not limited to, altretamine, aminoglutethimide, amsacrine, azacitidine, bleomycin, busulfan, carboplatin, chlorambucil, cisplatin, cyclophosphamide, cytarabine, decarbazine, dactinomycin, daunorubicin, doxorubicin, estramustine phosphate, ethinyl estradiol, floxuridine,
20   fludarabine phosphate, fluorouracil, flutamine, hydroxyurea, ifosfamide, lomustine, mechlorethamine, melphalan, mercaptopurine, methotrexate, mitomycin, mitotane, mitoxantrone, pentostatin, pipobroman, plicamycin, procarbazine, streptozocin, taxol, teniposide, thioguanine, triethylenethiophosphoramide, trimetrexate, uracil mustard, vinblastine, vincristine, and vindesine.
25

C.   Nucleic acid molecules

As noted above, nucleic acids may be modified with polyoxyalkylene. APO-nucleic acids are useful for administration to patients, such as in gene therapy protocols.   As used herein, "nucleic acids" refer to RNA, DNA, PNA and other
30   derivatives that are intended for administration to a patient. The nucleic acids include duplex DNA, single-stranded DNA, RNA in any form, including triplex, duplex or

single-stranded RNA, anti-sense RNA, polynucleotides, oligonucleotides; single nucleotides, chimeras, and derivatives thereof.

Nucleic acids may be composed of the well-known deoxyribonucleotides and ribonucleotides composed of the bases adenosine, cytosine, guanine, thymidine, and uridine. As well, various other nucleotide derivatives and non-phosphate backbones or phosphate-derivative backbones may be used. Methods for producing nucleic acids are well known (*see* Ausubel et al., *infra*; Sambrook et al., *infra*).

If the APO-nucleic acids cannot be prepared by direct modification of amino groups on the pyrimidine or purine rings, other options are available. For example, a thionocleoside can be incorporated. The thio group can be modified directly with APO or may serve as a site of attachment for a linker with an amino group (*i.e.*, Traut's reagent). Further, APO modification of other linkers attached via the heterocyclic rings or phosphate groups of the nucleic acid is possible.

D.   *Additional therapeutic agents*

Other therapeutic agents are also useful within the context of this invention. Such therapeutics include any medically relevant compound that has a suitable group available for modification or can be derivatized to contain a suitable group. Examples of therapeutics include, but are not limited to, analgesics, antidiabetic agents, viruses, antiarthritic compounds, anti-inflammatory compounds, hormones, cardioprotective agents, contraceptives, migraine preparations, psychotherapeutic agents, respiratory drugs, and the like. Derivatization, if necessary, to provide an amino or nucleophilic group is performed by standard chemical methods known to those skilled in the art

5.   THERAPEUTIC FORMULATIONS AND ADMINISTRATION

The present invention noted above, the present invention provides compositions for modifying therapeutics for treating and preventing diseases and syndromes by administering to a patient a therapeutically effective amount of an APO-modified therapeutic. Patients suitable for such treatment may be identified by well-established hallmarks.

Infections that may be treated with APO-peptides or APO-antibiotics include those caused by or due to microorganisms. Examples of microorganisms include bacteria (*e.g.*, Gram-positive, Gram-negative), fungi, (*e.g.*, yeast and molds), parasites (*e.g.*, protozoans, nematodes, cestodes and trematodes), viruses, and prions. Specific organisms in these classes are well known (*see* for example, Davis et al., *Microbiology*, 3rd edition, Harper & Row, 1980). Infections include, but are not limited

to, toxic shock syndrome, diphtheria, cholera, typhus, meningitis, whooping cough, botulism, tetanus, pyogenic infections, dysentery, gastroenteritis, anthrax, Lyme disease, syphilis, rubella, septicemia and plague. Effective treatment of infection may be examined in several different ways. The patient may exhibit reduced fever, reduced

5 number of organisms, lower level of inflammatory molecules (e.g., IFN-γ, IL-12, IL-1, TNF), and the like.

In gene therapy protocols, a nucleic acid encoding a desired product in introduced into a cell or cells. Many means for introduction of nucleic acids into cells are known. Such methods include retroviral vectors and subsequent retrovirus

10 infection, adenovirals or adeno-associated viral vectors and subsequent infection, complexes of nucleic acid with a condensing agent (e.g., poly-lysine), these complexes or viral vectors may be targeted to particular cell types by way of an incorporated ligand. A wide variety of vectors may be utilized within the context of the present invention, including for example, plasmids, viruses, retrotransposons and cosmids.

15 Representative examples include adenoviral vectors (e.g., WO 94/26914, WO 93/9191; Yei et al., Gene Therapy 1:192-200, 1994; Kolls et al., PNAS 91(1):215-219, 1994; Kass-Eisler et al., PNAS 90(24):11498-502, 1993; Guzman et al., Circulation 88(6):2838-48, 1993; Guzman et al., Cir. Res. 73(6):1202-1207, 1993; Zabner et al., Cell 75(2):207-216, 1993; Li et al., Hum Gene Ther. 4(4):403-409, 1993; Caillaud et

20 al., Eur. J. Neurosci. 5(10):1287-1291, 1993), adeno-associated type 1 ("AAV-1") or adeno-associated type 2 ("AAV-2") vectors (see WO 95/13365; Flotte et al., PNAS 90(22):10613-10617, 1993), hepatitis delta vectors, live, attenuated delta viruses and herpes viral vectors (e.g., U.S. Patent No. 5,288,641), as well as vectors which are disclosed within U.S. Patent No. 5,166,320. Other representative vectors include

25 retroviral vectors (e.g., EP 0 415 731; WO 90/07936; WO 91/02805; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218. In addition, many ligands specific for tumor cells and other cells are well known in the art.

Within the context of this invention, the gene delivery vehicle can be

30 APO-modified for administration. As noted above, the vehicle is generally nucleic acid, virus, or lipid. Any of these can be modified using the procedures disclosed herein. Within certain aspects of the invention, gene delivery vehicles may be introduced into a host cell utilizing a vehicle or by various physical methods. Representative examples of such methods include transformation using calcium

35 phosphate precipitation (Dubensky et al., PNAS 81:7529-7533, 1984), direct microinjection of such nucleic acid molecules into intact target cells (Acsadi et al., Nature 352:815-818, 1991), and electroporation whereby cells suspended in a conducting solution are subjected to an intense electric field in order to transiently

polarize the membrane, allowing entry of the nucleic acid molecules. Other procedures include the use of nucleic acid molecules linked to an inactive adenovirus (Cotton et al., *PNAS 89*:6094, 1990), lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991), polycation compounds such as polylysine, receptor specific ligands, liposomes entrapping the nucleic acid molecules, spheroplast fusion whereby *E. coli* containing the nucleic acid molecules are stripped of their outer cell walls and fused to animal cells using polyethylene glycol, viral transduction, (Cline et al., *Pharmac. Ther. 29*:69, 1985; and Friedmann et al., *Science 244*:1275, 1989), and DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 1989), as well as psoralen inactivated viruses such as Sendai or Adenovirus.

The modified therapeutics of the present invention are preferably administered as a pharmaceutical composition. Briefly, pharmaceutical compositions of the present invention may comprise one or more of the APO-therapeutics described herein, in combination with one or more pharmaceutically acceptable carriers, diluents, or excipients. As noted herein, the formulation buffer used may affect the efficacy or activity of the peptide analogue. A suitable formulation buffer contains buffer and solubilizer. The formulation buffer may comprise buffers such as sodium acetate, sodium citrate, neutral buffered saline, phosphate-buffered saline, and the like or salts, such as NaCl. Sodium acetate is preferred. In general, an acetate buffer from 5 to 500mM is used, and preferably from 100 to 200 mM. The pH of the final formulation may range from 3 to 10, and is preferably approximately neutral (about pH 7-8). Solubilizers, such as polyoxyethylenesorbitans (*e.g.*, Tween 80, Tween 20) and polyoxyethylene ethers (*e.g.*, Brij 56) may also be added if the compound is not already APO-modified. Although the formulation buffer is exemplified herein with peptide of the present invention, this buffer is generally useful and desirable for delivery of other therapeutics.

Additional compounds may be included in the compositions. These include, for example, carbohydrates such as glucose, mannose, sucrose or dextrose, mannitol, other proteins, polypeptides or amino acids, chelating agents such as EDTA or glutathione, adjuvants and preservatives. As noted herein, pharmaceutical compositions of the present invention may also contain one or more additional active ingredients, such as an antibiotic or cytokine.

The compositions may be administered in a delivery vehicle. For example, the composition can be encapsulated in a liposome (*see, e.g.*, WO 96/10585; WO 95/35094), complexed with lipids, encapsulated in slow-release or sustained release vehicles, such as poly-galactide, and the like. Within other embodiments,

compositions may be prepared as a lyophilizate, utilizing appropriate excipients to provide stability.

Pharmaceutical compositions of the present invention may be administered in various manners, by intravenous injection, intraperitoneal injection or implantation, subcutaneous injection or implantation, intradermal injection, lavage, inhalation, implantation, intramuscular injection or implantation, intrathecal injection, bladder wash-out, suppositories, pessaries, topical (e.g., creams, ointments, skin patches, eye drops, ear drops, shampoos) application, enteric, oral, or nasal route. The modified therapeutic may be applied locally as an injection, drops, spray, tablets, cream, ointment, gel, and the like. The therapeutic may be administered as a bolus or as multiple doses over a period of time.

The level of therapeutic in serum and other tissues after administration can be monitored by various well-established techniques such as bacterial, chromatographic or antibody based, such as ELISA, and the like.

Pharmaceutical compositions of the present invention are administered in a manner appropriate to the infection or disease to be treated. The amount and frequency of administration will be determined by factors such as the condition of the patient, the cause of the infection or disease, and the severity of the infection or disease. Appropriate dosages may be determined by clinical trials.

For these purposes, typically the therapeutics are included in compositions commonly employed or in a suitable applicator, such as for applying to clothing. They may be incorporated or impregnated into the material during manufacture, such as for an air filter, or otherwise applied to devices. The therapeutics need only be suspended in a solution appropriate for the device or article. Polymers are one type of carrier that can be used.

The modified compounds, especially the labeled ones, may be used in image analysis and diagnostic assays or for targeting sites in eukaryotic multicellular and single cell cellular organisms and in prokaryotes. As a targeting system, the modified compounds may be coupled with other peptides, proteins, nucleic acids, antibodies and the like.

In one aspect of the present invention, cationic peptides are used to treat tumors. Suitable tumor targets include, for example, lymphoma, leukemia, multiple myeloma, breast, lung, ovarian, cervical, uterine, skin, prostate, liver, and colon cancer. Cationic peptides can be administered as antineoplastic agents by methods described above. Preferred modes of administration include intravenous, intraperitoneal, intramuscular, subcutaneous, and intralesional administration.

Cationic peptides, modified or unmodified, can be administered alone, or in conjugation with a conventional antineoplastic agent. Useful antineoplastic drugs

include nitrogen mustards, alkyl sulfonates, nitrosoureas, triazenes, folic acid analogs, pyrimidine analogs, purine analogs, epipodophyllotoxins, platinum coordination complexes, hormones, and the like. Additional suitable antineoplastic drugs are described in Gennaro (Ed.), *Remington: The Science and Practice of Pharmacy, 19th*

5   *Edition,* (Mack Publishing Co. 1995), and in Gilman et al. (Eds.), *Goodman and Gilman's The Pharmacological Basis of Therapeutics, 7th Ed.* (MacMillan Publishing Co. 1985). Other suitable antineoplastic drugs, such as experimental drugs, are known to those of skill in the art. Modes of antineoplastic drug administration and suitable dosages of antineoplastic drugs are well known to those of skill in the art (see, for

10   example, Gennaro (Ed.), *Remington: The Science and Practice of Pharmacy, 19th Edition,* (Mack Publishing Co. 1995), and Gilman et al. (Eds.), *Goodman and Gilman's The Pharmacological Basis of Therapeutics, 7th Ed.* (MacMillan Publishing Co. 1985)). An advantage of a therapy regimen that includes administration of an antineoplastic drug and at least one cationic peptide is that a lower dosage of the

15   antineoplastic drug is required due the synergistic effect of cationic peptides and antineoplastic drugs.

Although cationic peptides are effective against multidrug resistant tumor cells, it may be desirable to supplement therapy with a "chemosensitizing agent" that reverses the multidrug resistance phenotype. Useful chemosensitizing agents

20   include verapamil and its analogs, calmodulin antagonists, anthracycline, and *Vinca* alkaloid analogs, and the like (see, for example, Endicott *et al., Ann. Rev. Biochem. 58*:137, 1989, Ford *et al., Pharmacol. Rev. 42*:155, 1990, Calabresi *et al., PPO Updates 8*:1, 1994, and Sarkadi *et al., FASEB J. 8*:766, 1994). Chemosensitizing agents may be administered prior to, or concurrent with, the administration of cationic peptides.

25   Typical modes of administration and dosages of chemosensitizing agents are described, for example, by Presant *et al., Am. J. Clin. Oncol. 9*:355, 1986, Cairo *et al., Cancer Res. 49*:1063, 1989, Miller *et al., J. Clin. Oncol. 9*:37, 1991, Rubin, U.S. Patent No. 5,005,588, and Levy, U.S. Patent No. 5,258,372.

For purposes of therapy, a cationic peptide and a pharmaceutically

30   acceptable carrier are administered to a subject in a therapeutically effective amount. Such a pharmaceutical composition is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient subject. In the present context, an agent is physiologically significant if its

35   presence results in the inhibition of the growth of target cells, or in the increased susceptibility of target cells to an antibiotic.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

APPLICATION TEMPLATE AND STYLES ATTACHED (SEE WP FOR

5 *ASSISTANCE)*

EXAMPLES

EXAMPLE 1

ACTIVATION OF POLYSORBATE 80 BY ULTRAVIOLET LIGHT

A solution of 2% (w/w) polysorbate 80 is prepared in water and 200ml

10 are placed in a 250mL crystallizing dish or over suitable container. Containers must have a clear light path. Cover the vessel with a piece of UV transparent plastic wrap or other UV transparent material. In addition, the material should allow the exchange of air but minimize evaporation.

The solution is irradiated with ultraviolet light using a lamp emitting at

15 254 nm. Irradiation can also be performed using a lamp emitting at 302 nm. The solution should be stirred continuously to maximize the rate of activation. The activation is complete within 72 hours using a lamp with a output of $1800\mu W/cm^2$. The reaction is monitored by a reversed-phased HPLC assay, which measures the formation of APO-MBI 11CN-Tw80 when the light-activated polysorbate is reacted with

20 MBI 11CN.

Some properties of activated polysorbate are determined. Because peroxides are a known by-product of exposing ethers to UV light, peroxide formation is examined through the effect of reducing agents on the activated polysorbate. As seen in Figure 1, graph a, activated polysorbate readily reacts with MBI 11CN. Pre-

25 treatment with 2-mercaptoethanol (Figure 1, graph b), a mild reducing agent, eliminates detectable peroxides, but does not cause a loss of conjugate forming ability. Treatment with sodium borohydride (Figure 1, graph b), eliminates peroxides and eventually eliminates the ability of activated polysorbate to modify peptides. Hydrolysis of the borohydride in water raises the pH and produces borate as a hydrolysis product.

30 However, neither a pH change nor borate are responsible.

These data indicate that peroxides are not involved in the modification of peptides by activated polysorbate. Sodium borohydride should not affect epoxides or esters in aqueous media, suggesting that the reactive group is an aldehyde or ketone. The presence of aldehydes in the activated polysorbate is confirmed by using a

35 formaldehyde test, which is specific for aldehydes including aldehydes other than formaldehyde.

Furthermore, activated polysorbate is treated with 2,4-dinitrophenylhydrazine (DNPH) in an attempt to capture the reactive species. Three

DNPH-tagged components are purified and analyzed by mass spectroscopy. These components are polysorbate-derived with molecular weights between 1000 and 1400. This indicates that low molecular weight aldehydes, such as formaldehyde or acetaldehyde, are involved.

EXAMPLE 2

ACTIVATION OF POLYSORBATE 80 BY AMMONIUM PERSULFATE

A 200 mL solution of 2% (w/w) polysorbate 80 is prepared in water. To this solution, 200 mg of ammonium persulfate is added while stirring. The reaction is stirred for 1-2 hours with protection from ambient light. If a solution of less than 0.1% (w/w) ammonium persulfate is used, then exposure to ultraviolet light at 254 nm during this period is used to help complete the reaction. The peroxide level in the reaction is determined using a test kit. Peroxides are reduced by titration with 2-mercaptoethanol.

EXAMPLE 3

FORMATION OF APO-MODIFIED PEPTIDES

APO-modified peptides are prepared either in solid phase or liquid phase. For solid phase preparation, 0.25 ml of 4 mg/ml of MBI 11CN is added to 0.5 ml of 0.4 M Acetic acid-NaOH pH 4.6 followed by addition of 0.25ml of UV-activated polysorbate. The reaction mix is frozen by placing it in a -80°C freezer. After freezing, the reaction mix is lyophilized overnight.

For preparing the conjugates in an aqueous phase, a sample of UV activated polysorbate 80 is first adjusted to a pH of 7.5 by the addition of 0.1M NaOH. This pH adjusted solution (0.5 ml) is added to 1.0 ml of 100 mM sodium carbonate, pH 10.0, followed immediately by the addition of 0.5 ml of 4 mg/ml of MBI 11CN. The reaction mixture is incubated at ambient temperature for 22 hours. The progress of the reaction is monitored by analysis at various time points using RP-HPLC (Figure 2). In Figure 2, peak 2 is unreacted peptide, peak 3 is APO-modified peptide. Type 1 is the left-most of peak 3 and Type 2 is the right-most of peak 3.

Polysorbate 80 (TWEEN 80) at 2% (w/w) in water is activated. Ammonium persulfate (AP) is present at 0.05% in the AP and the AP + UV samples. The UV and AP + UV samples are exposed to ultraviolet light at 254 nm using a lamp with an output of 1750-2000 $\mu W/cm^2$ during the time period. The reaction is stirred continuously with a magnetic stirrer at 100-200 rpm. Aliquots are removed and stored in the dark at -80°C until assayed. Aliquots are reacted with MBI11CN using the lyophilization method and the generation of MBI11CN-Tw80 was measured by RP-HPLC (Figure 2B).

The table below summarizes data from several experiments. Unless otherwise noted in the table, the APO-modified peptides are prepared via the lyophilization method in 200mM acetic acid-NaOH buffer, pH 4.6.

5

Table 4

SEQUENCE	NAME	COMPLEX	
		TYPE 1	TYPE 2
ILKKWPWWPWRRKamide	11CN		
Solid phase, pH 2.0		Yes	Low
Solid phase, pH 4.6		Yes	Yes
Solid phase, pH 5.0		Yes	Yes
Solid phase, pH 6.0		Yes	Yes
Solid phase, pH 8.3		Yes	Yes
Solution, pH 2.0		Trace	Trace
Solution, pH 10.0		Yes	Yes-Slow
(Ac)₄-ILKKWPWWPWRRKamide	11CN-Y1	No	No
ILRRWPWWPWRRKamide	11B1CN	Yes	Lowered
ILRWPWWPWRRKamide	11B7CN	Yes	Lowered
ILWPWWPWRRKamide	11B8CN	Yes	Lowered
ILRRWPWWPWRRRamide	11B9CN	Yes	Trace
ILKKWPWWPWKKKamide	11B10CN	Yes	Yes
iLKKWPWWPWRRkamide	11E3CN	Yes	Yes
ILKKWVWWPWRRKamide	11F3CN	Yes	Yes
ILKKWPWWPWKamide	11G13CN	Yes	Yes
ILKKWPWWPWRamide	11G14CN	Yes	Trace

The modification of amino groups is further analyzed by determining the number of primary amino groups lost during attachment. The unmodified and modified
10 peptides are treated with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (R.L. Lundblad in *Techniques in Protein Modification and Analysis* pp. 151-154, 1995).

Briefly, a stock solution of MBI 11CN at 4 mg/ml and an equimolar solution of APO-modified MBI 11CN are prepared. A 0.225 ml aliquot of MBI 11CN or APO-modified MBI 11CN is mixed with 0.225 ml of 200 mM sodium phosphate
15 buffer, pH 8.8. A 0.450 ml aliquot of 1% TNBS is added to each sample, and the

reaction is incubated at 37°C for 30 minutes. The absorbance at 367 nm is measured, and the number of modified primary amino groups per molecule is calculated using an extinction coefficient of 10,500 M^{-1} cm^{-1} for the trinitrophenyl (TNP) derivatives.

5 The primary amino group content of the parent peptide is then compared to the corresponding APO-modified peptide. As shown below, the loss of a single primary amino group occurs during formation of modified peptide. Peptides possessing a 3,4 lysine pair consistently give results that are 1 residue lower than expected, which may reflect steric hindrance after titration of one member of the doublet.

10 **Table 5**

PEPTIDE SEQUENCE	TNP/PEPTIDE	TNP/APO-modified peptide	CHANGE
ILKKWPWWPWRRKamide	2.71	1.64	1.07
ILRRWPWWPWRRKamide	1.82	0.72	1.10
IIKKWPWWPWRRKamide	2.69	1.61	1.08
ILKKWVWWPWRRKamide	2.62	1.56	1.06

Stability of APO-modified peptide analogues

APO-modified peptides demonstrate a high degree of stability under
15 conditions that promote the dissociation of ionic or hydrophobic complexes. APO-modified peptide in formulation D is prepared as 800 μg/ml solutions in water, 0.9% saline, 8M urea, 8M guanidine-HCl, 67% 1-propanol, 1M HCl and 1M NaOH and incubated for 1 hour at room temperature. Samples are analyzed for the presence of free peptide using reversed phase HPLC and the following chromatographic conditions:

20   Solvent A: 0.1% trifluoroacetic acid (TFA) in water
                Solvent B: 0.1% TFA / 95% acetonitrile in water
                Media: POROS R2-20 (polystyrene divinylbenzene)
                Elution: 0% B for 5 column volumes
                         0-25% B in 3 column volumes
25   25% B for 10 column volumes
                         25-95% B in 3 column volumes
                         95% B for 10 column volumes

Under these conditions, free peptide elutes exclusively during the 25% B step and formulation-peptide complex during the 95% B step. None of the dissociating
30 conditions mentioned above, with the exception of 1M NaOH in which some degradation is observed, are successful in liberating free peptide from APO-modified

peptide. Additional studies are carried out with incubation at 55°C or 85°C for one hour. APO-modified peptide is equally stable at 55°C and is only slightly less stable at 85°C. Some acid hydrolysis, indicated by the presence of novel peaks in the HPLC chromatogram, is observed with the 1M HCl sample incubated at 85°C for one hour.

EXAMPLE 4

PURIFICATION OF APO-MODIFIED MBI 11CN

A large scale preparation of APO-modified MBI 11CN is purified. Approximately 400 mg of MBI 11CN is APO-modified and dissolved in 20ml of water. Unreacted MBI 11CN is removed by RP-HPLC. The solvent is then evaporated from the APO-modified MBI 11CN pool, and the residue is dissolved in 10 ml methylene chloride. The modified peptide is then precipitated with 10 ml diethyl ether. After 5 min at ambient temperature, the precipitate is collected by centrifugation at 5000xg for 10 minutes. The pellet is washed with 5 ml of diethyl ether and again collected by centrifugation at 5000xg for 10 minutes. The supernatants are pooled for analysis of unreacted polysorbate by-products. The precipitate is dissolved in 6 ml of water and then flushed with nitrogen by bubbling for 30 minutes to remove residual ether. The total yield from the starting MBI 11CN was 43%.

The crude APO-MBI29-Tw80 prepared from 200 mg of MBI 29 is suspended in 40mL of methylene chloride and sonicated for 5 minutes to disperse large particles. The suspension is centrifuged in appropriate containers (Corning glass) at 3000-4000 x g for 15 minutes at 10°C to sediment insoluble material. The supernatant is decanted and saved.

The sediment is extracted twice more by adding 40 mL portions methylene chloride to the sediment and repeating the sonication/centrifugation step. The supernatants from the three extractions are pooled and concentrated 8-10 fold using a rotary evaporator. The solution is transferred to centrifuge tubes and 3 volumes of diethyl ether are added. The mixture is incubated for 15 minutes, then centrifuged at 3000-4000 x g for 15 minutes at 10°C to sediment the product. The supernatant is decanted and discarded. The residual ether may be removed with a stream of nitrogen.

EXAMPLE 5

IN VITRO ASSAYS TO MEASURE APO-CATIONIC PEPTIDE ACTIVITY
Agarose Dilution Assay

The agarose dilution assay measures antimicrobial activity of peptides and peptide analogues, which is expressed as the minimum inhibitory concentration (MIC) of the peptides.

In order to mimic *in vivo* conditions, calcium and magnesium supplemented Mueller Hinton broth is used in combination with a low EEO agarose as the bacterial growth medium. The more commonly used agar is replaced with agarose as the charged groups in agar prevent peptide diffusion through the media. The media

5　is autoclaved and then cooled to 50 - 55° C in a water bath before aseptic addition of antimicrobial solutions. The same volume of different concentrations of peptide solution are added to the cooled molten agarose that is then poured to a depth of 3 - 4 mm.

The bacterial inoculum is adjusted to a 0.5 McFarland turbidity standard

10　(PML Microbiological) and then diluted 1:10 before application on to the agarose plate. The final inoculum applied to the agarose is approximately 10^4 CFU in a 5 - 8 mm diameter spot. The agarose plates are incubated at 35 - 37°C for 16 to 20 hours.

The MIC is recorded as the lowest concentration of peptide that completely inhibits growth of the organixm as determined by visual inspection.

15　Representative MICs for various modified and unmodified cationic peptides are shown in Table 6.

Table 6

Organism	Organism #	APO-Peptide	Corrected MIC(µg/mL)	
			APO-Peptide	Peptide
A. calcoaceticus	AC002	MBI11CN-Tw80	4	4
A. calcoaceticus	AC002	MBI11B1CN-Tw80	4	2
A. calcoaceticus	AC002	MBI11B7CN-Tw80	4	2
A. calcoaceticus	AC002	MBI11B7CN-Tx114r	2	2
A. calcoaceticus	AC002	MBI11B7CN-F12-Tx114r	1	1
A. calcoaceticus	AC002	MBI11E3CN-Tw80	2	1
A. calcoaceticus	AC002	MBI11F3CN-Tw80	8	2
A. calcoaceticus	AC002	MBI11F4CN-Tw80	4	4
A. calcoaceticus	AC002	MBI29-Tw80	4	1
E. cloacae	ECL007	MBI11CN-Tw80	>128	>128
E. cloacae	ECL007	MBI11B1CN-Tw80	128	>128
E. cloacae	ECL007	MBI11B7CN-Tw80	>128	128
E. cloacae	ECL007	MBI11B7CN-Tx114r	128	128
E. cloacae	ECL007	MBI11B7CN-F12-Tx114r	>128	>128
E. cloacae	ECL007	MBI11E3CN-Tw80	128	>128
E. cloacae	ECL007	MBI11F3CN-Tw80	128	>128
E. cloacae	ECL007	MBI11F4CN-Tw80	64	32

E. cloacae	ECL007	MBI29-Tw80	32	>64
E.coli	ECO005	MBI11CN-Tw80	16	8
E.coli	ECO005	MBI11B1CN-Tw80	8	8
E.coli	ECO005	MBI11B7CN-Tw80	16	4
E.coli	ECO005	MBI11B7CN-Tx114r	16	4
E.coli	ECO005	MBI11B7CN-F12-Tx114r	32	16
E.coli	ECO005	MBI11E3CN-Tw80	8	4
E. coli	ECO005	MBI11F3CN-Tw80	128	16
E. coli	ECO005	MBI11F4CN-Tw80	8	8
E.coli	ECO005	MBI29-Tw80	16	4
E. faecalis	EFS001	MBI11CN-Tw80	8	32
E. faecalis	EFS001	MBI11B1CN-Tw80	4	32
E. faecalis	EFS001	MBI11B7CN-Tw80	8	8
E. faecalis	EFS001	MBI11B7CN-Tx114r	0.5	0.5
E. faecalis	EFS001	MBI11B7CN-F12-Tx114r	0.5	0.5
E. faecalis	EFS001	MBI11E3CN-Tw80	4	8
E. faecalis	EFS001	MBI11F3CN-Tw80	8	32
E. faecalis	EFS001	MBI29-Tw80	0.5	0.5
E. faecalis	EFS004	MBI11CN-Tw80	4	8
E. faecalis	EFS004	MBI11B1CN-Tw80	4	8
E. faecalis	EFS004	MBI11B7CN-Tw80	8	8
E. faecalis	EFS004	MBI11E3CN-Tw80	4	2
E. faecalis	EFS004	MBI11F3CN-Tw80	4	16
E. faecalis	EFS008	MBI11CN-Tw80	1	16
E. faecalis	EFS008	MBI11B1CN-Tw80	1	2
E. faecalis	EFS008	MBI11B7CN-Tw80	1	2
E. faecalis	EFS008	MBI11B7CN-Tx114r	2	4
E. faecalis	EFS008	MBI11B7CN-F12-Tx114r	2	2
E. faecalis	EFS008	MBI11E3CN-Tw80	1	2
E. faecalis	EFS008	MBI11F3CN-Tw80	4	16
E. faecalis	EFS008	MBI11F4CN-Tw80	2	2
E. faecalis	EFS008	MBI29-Tw80	2	0.5
K. pneumoniae	KP001	MBI11CN-Tw80	8	16
K. pneumoniae	KP001	MBI11B1CN-Tw80	8	8
K. pneumoniae	KP001	MBI11B7CN-Tw80	8	4
K. pneumoniae	KP001	MBI11B7CN-Tx114r	8	8
K. pneumoniae	KP001	MBI11B7CN-F12-Tx114r	32	16

K. pneumoniae	KP001	MBI11E3CN-Tw80	4	8
K. pneumoniae	KP001	MBI11F3CN-Tw80	128	64
K. pneumoniae	KP001	MBI11F4CN-Tw80	8	4
K. pneumoniae	KP001	MBI29-Tw80	16	2
P. aeruginosa	PA004	MBI11CN-Tw80	>128	128
P. aeruginosa	PA004	MBI11B1CN-Tw80	128	64
P. aeruginosa	PA004	MBI11B7CN-Tw80	128	128
P. aeruginosa	PA004	MBI11B7CN-Tx114r	128	128
P. aeruginosa	PA004	MBI11B7CN-F12-Tx114r	>128	>128
P. aeruginosa	PA004	MBI11E3CN-Tw80	64	32
P. aeruginosa	PA004	MBI11F3CN-Tw80	128	128
P. aeruginosa	PA004	MBI11F4CN-Tw80	128	32
P. aeruginosa	PA004	MBI29-Tw80	>64	16
S. aureus	SA010	MBI11B1CN	4	1
S. aureus	SA010	MBI11B7CN	4	1
S. aureus	SA010	MBI11CN	4	2
S. aureus	SA010	MBI11E3CN	2	1
S. aureus	SA010	MBI11F3CN	4	2
S. aureus	SA011	MBI11CN-Tw80	16	8
S. aureus	SA011	MBI11B1CN-Tw80	16	4
S. aureus	SA011	MBI11B7CN-Tw80	16	4
S. aureus	SA011	MBI11E3CN-Tw80	16	4
S. aureus	SA011	MBI11F3CN-Tw80	16	8
S. aureus	SA014	MBI11CN-Tw80	2	1
S. aureus	SA014	MBI11B1CN-Tw80	2	1
S. aureus	SA014	MBI11B7CN-Tw80	1	2
S. aureus	SA014	MBI11B7CN-Tx114r	2	1
S. aureus	SA014	MBI11B7CN-F12-Tx114r	2	2
S. aureus	SA014	MBI11E3CN-Tw80	1	1
S. aureus	SA014	MBI11F3CN-Tw80	8	8
S. aureus	SA014	MBI11F4CN-Tw80	2	2
S. aureus	SA014	MBI29-Tw80	2	1
S. aureus	SA018	MBI11CN-Tw80	64	64
S. aureus	SA018	MBI11B1CN-Tw80	32	16
S. aureus	SA018	MBI11B7CN-Tw80	32	16
S. aureus	SA018	MBI11E3CN-Tw80	32	16
S. aureus	SA018	MBI11F3CN-Tw80	64	16

S. aureus	SA025	MBI11CN-Tw80	2	4
S. aureus	SA025	MBI11B1CN-Tw80	4	1
S. aureus	SA025	MBI11B7CN-Tw80	2	1
S. aureus	SA025	MBI11E3CN-Tw80	2	1
S. aureus	SA025	MBI11F3CN-Tw80	4	2
S. aureus	SA093	MBI11CN-Tw80	2	2
S. aureus	SA093	MBI11B1CN-Tw80	2	1
S. aureus	SA093	MBI11B7CN-Tw80	2	1
S. aureus	SA093	MBI11B7CN-Tx114r	1	1
S. aureus	SA093	MBI11B7CN-F12-Tx114r	1	1
S. aureus	SA093	MBI11E3CN-Tw80	2	1
S. aureus	SA093	MBI11F3CN-Tw80	2	1
S. aureus	SA093	MBI29-Tw80	1	0.5
S. epidermidis	SE010	MBI11B7CN-Tx114r	4	2
S. epidermidis	SE010	MBI11B7CN-F12-Tx114r	4	8
S. epidermidis	SE010	MBI29-Tw80	>64	4
S. maltophilia	SMA002	MBI11CN-Tw80	32	>128
S. maltophilia	SMA002	MBI11B1CN-Tw80	32	32
S. maltophilia	SMA002	MBI11B7CN-Tw80	64	16
S. maltophilia	SMA002	MBI11B7CN-Tx114r	32	16
S. maltophilia	SMA002	MBI11B7CN-F12-Tx114r	64	64
S. maltophilia	SMA002	MBI11E3CN-Tw80	128	64
S. maltophilia	SMA002	MBI11F3CN-Tw80	128	64
S. maltophilia	SMA002	MBI11F4CN-Tw80	32	16
S. maltophilia	SMA002	MBI29-Tw80	8	2
S. marcescens	SMS003	MBI11CN-Tw80	>128	>128
S. marcescens	SMS003	MBI11B1CN-Tw80	>128	>128
S. marcescens	SMS003	MBI11B7CN-Tw80	>128	>128
S. marcescens	SMS003	MBI11B7CN-Tx114r	>128	>128
S. marcescens	SMS003	MBI11B7CN-F12-Tx114r	>128	>128
S. marcescens	SMS003	MBI11E3CN-Tw80	128	>128
S. marcescens	SMS003	MBI11F3CN-Tw80	128	>128
S. marcescens	SMS003	MBI11F4CN-Tw80	>128	>128
S. marcescens	SMS003	MBI29-Tw80	>64	>128

Broth Dilution Assay

This assay also uses calcium- and magnesium supplemented Mueller Hinton broth as the growth medium. Typically 100 µl of broth is dispensed into each well of a 96-well microtitre plate and 100 µl volumes of two-fold serial dilutions of the peptide analogue are made across the plate. One row of wells receives no peptide and is used as a growth control. Each well is inoculated with approximately 5 x 10⁵ CFU of bacteria and the plate is incubated at 35 - 37°C for 16-20 hours. The MIC is again recorded at the lowest concentration of peptide that completely inhibits growth of the organism as determined by visual inspection.

Time Kill Assay

Time kill curves are used to determine the antimicrobial activity of cationic peptides over a time interval. Briefly, in this assay, a suspension of microorganisms equivalent to a 0.5 McFarland Standard is prepared in 0.9% saline. This suspension is then diluted such that when added to a total volume of 9 ml of cation-adjusted Mueller Hinton broth, the inoculum size is 1 x 10⁶ CFU/ml. An aliquot of 0.1 ml is removed from each tube at pre-determined intervals up to 24 hours, diluted in 0.9% saline and plated in triplicate to determine viable colony counts. The number of bacteria remaining in each sample is plotted over time to determine the rate of cationic peptide killing. Generally a three or more $\log_{10}$ reduction in bacterial counts in the antimicrobial suspension compared to the growth controls indicate an adequate bactericidal response.

As shown in Figure 3, all peptides demonstrated a three or more $\log_{10}$ reduction in bacterial counts in the antimicrobial suspension compared to the growth controls indicating that these peptides have met the criteria for a bactericidal response.

Synergy Assay

Treatment with a combination of peptide analogues and conventional antibiotics can have a synergistic effect. Synergy is assayed using the agarose dilution technique, where an array of plates, each containing a combination of peptide and antibiotic in a unique concentration mix, is inoculated with the bacterial isolates. Synergy is investigated for peptide analogues in combination with a number of conventional antibiotics including, but not limited to, penicillins, cephalosporins, carbapenems, monobactams, aminoglycosides, macrolides, fluoroquinolones.

Synergy is expressed as a Fractional Inhibitory Concentration (FIC), which is calculated according to the equation below. An FIC of less than or equal to 0.5 is evidence of synergy, although combinations with higher values may be therapeutically useful.

$$FIC = \frac{MIC \ (peptide \ in \ combination)}{MIC \ (peptide \ alone)} + \frac{MIC \ (antibiotic \ in \ combination)}{MIC \ (antibiotic \ alone)}$$

5   <u>EXAMPLE 6</u>

BIOLOGICAL ASSAYS USING APO-MODIFIED PEPTIDE

All biological assays that compare APO-modified peptides with unmodified peptides are performed on an equimolar ratio. The concentration of APO-modified peptides can be determined by spectrophotometric measurement, which is
10 used to normalize concentrations for biological assays. For example, a 1 mg/ml APO-modified MBI 11CN solution contains the same amount of peptide as a 1 mg/ml MBI 11CN solution, thus allowing direct comparison of toxicity and efficacy data.

APO-modified peptides are at least as potent as the parent peptides in *in vitro* assays performed as described herein. MIC values against gram positive bacteria
15 are presented for several APO-modified peptides and compared with the values obtained using the parent peptides (Table 5). The results indicate that the modified peptides are at least as potent *in vitro* as the parent peptides and may be more potent than the parent peptides against *E. faecalis* strains.

Toxicities of APO-modified MBI 11CN and unmodified MBI 11CN are
20 examined in Swiss CD-1 mice. Groups of 6 mice are injected iv with single doses of 0.1 ml peptide in 0.9% saline. The dose levels used are 0, 3, 5, 8, 10, and 13 mg/kg. Mice are monitored at 1, 3, and 6 hrs post-injection for the first day, then twice daily for 4 days. The survival data for MBI 11CN mice are presented in Table 7. For APO-modified MBI 11CN, 100% of the mice survived at all doses, including the maximal
25 dose of 13 mg/kg.

Table 7

Peptide administered (mg/kg)	No. Dead/Total	Cumulative Dead	No. Surviving	Cumulative No. Dead/Total	% Dead
13	6/6	18	0	18/18	100
10	6/6	12	0	12/12	100
8	6/6	6	0	6/6	100
5	0/6	0	6	0/6	0
3	0/6	0	12	0/12	0
0	0/6	0	18	0/18	0

As summarized below, the LD$_{50}$ for MBI 11CN is 7 mg/kg (Table 8), with all subjects dying at a dose of 8 mg/ml. The highest dose of MBI 11CN giving 100% survival was 5 mg/kg. The data show that APO-modified peptides are significantly less toxic than the parent peptides.

Table 8

Test Peptide	LD$_{50}$	LD$_{90-100}$	MTD
MBI-11CN-TFA	7 mg/kg	8 mg/kg	5 mg/kg
APO-MBI-11CN	> 13 mg/kg*	>13 mg/kg*	>13 mg/kg*

* could not be calculated with available data.

In addition, APO-peptides and parent peptides are tested against a panel of cancer cell lines. Cell death is measured using the Cytotox (Promega) assay kit which measures the release of lactate dehydrogenase. As shown below the modified peptides had increased activity over the parent peptides.

Table 9

Peptide	CELL LINE, LC$_{50}$, μg/mL±S.E.								
	PBL	HUVEC	H460	K562	DoHH-2	P388	P388ADR	MCF-7	MCF-7ADR
11CN	57	>190	200	-----	-----	30	25	11.8±9	17±1
11CN-Tw80	6±6	16±4	16±4	-----	-----	1.9±5	3.5±2	11	-----
11A3CN	>500	>500	>500	>500	>500	>300	>300	----	-----
11A3CN-Tw80	12.7±15	17±9	15±4	6	3.3±0.05	5.6±2	6.6±3	28	13
11B7CN	24±10	90±23	26±25	34±25	16.5±3	13.8	-----	>700	-----
11B7CN-Tw80	3.8±1	12.8±8	>100	4.7±3	3.3±1	5.1	-----	12	-----
11E3CN	22±11	117±7	18	9	3.6	13.9±3	7.9±3	5.6±2	5.3±1
11E3CN-Tw80	4.5±2	12.8±2	8.2±4	4.9±3	3.5±0.7	5.9±3	8.4±1	8.1±5	7.6±2
21A11	30±15	184±100	48	56±33	9.8±0.3	-----	-----	-----	----
21A11-	4.5±4	17±9.9	21	4.3±2	4.7±0.6	8.1±3.	9	18	----

Tw80						4			
29	12±10	10	12.6±10	1	2.1±0.5	1.4±0.5	2±0.2	4±2	3.2±1
29-Tw80	8.7±6	9.3±2	1.7	2.1±0.5	4±0.5	7.6±2.4	7.6±2	15.5±6	9.1±5

PBL, peripheral blood lymphocytes; HUVEC, human umbilical vein endothelial cells; H460, non-small lung tumor; K562, chronic myelogenous leukemia; DoHH-2, B-cell cell lymphoma; P388, lymphocytic leukemia; P388ADR, lymphocytic leukemia, multidrug resistant; MCF-7, breast carcinoma; MCF-7ADR, breast carcinoma, multidrug resistant.

EXAMPLE 7

SYNTHESIS, PURIFICATION, AND CHARACTERIZATION OF PEPTIDES

Peptide synthesis is based on the standard solid-phase Fmoc protection strategy. The instrument employed is a 9050 Plus PepSynthesiser (PerSeptive BioSystems Inc.). Polyethylene glycol polystyrene (PEG-PS) graft resins are employed as the solid phase, derivatized with an Fmoc-protected amino acid linker for C-terminal amide synthesis. HATU (O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) is used as the coupling reagent. During synthesis, coupling steps are continuously monitored to ensure that each amino acid is incorporated in high yield. The peptide is cleaved from the solid-phase resin using trifluoroacetic acid and appropriate scavengers and the crude peptide is purified using preparative reversed-phase chromatography.

All peptides are analyzed by mass spectrometry to ensure that the product has the expected molecular mass. The product should have a single peak accounting for >95% of the total peak area when subjected to analytical reversed-phase high performance liquid chromatography (RP-HPLC). In addition, the peptide should show a single band accounting for >90% of the total band intensity when subjected to acid-urea gel electrophoresis.

Peptide content, the amount of the product that is peptide rather than retained water, salt or solvent, is measured by quantitative amino acid analysis, free amine derivatization or spectrophotometric quantitation. Amino acid analysis also provides information on the ratio of amino acids present in the peptide, which assists in confirming the authenticity of the peptide. Peptides may be modified to alter the physical properties of the original peptide.

EXAMPLE 8

STRUCTURAL ANALYSIS OF APO-INDOLICIDIN VARIANTS USING CIRCULAR DICHROISM SPECTROSCOPY

5   Circular dichroism (CD) is a spectroscopic technique that measures secondary structures of peptides and proteins in solution, see for example, R.W. Woody, (*Methods in Enzymology, 246*: 34, 1995). The CD spectra of α-helical peptides is most readily interpretable due to the characteristic double minima at 208 and 222 nm. For peptides with other secondary structures however, interpretation of CD

10   spectra is more complicated and less reliable. The CD data for peptides is used to relate solution structure to *in vitro* activity.

CD measurements of indolicidin analogues are performed in three different aqueous environments, (1) 10 mM sodium phosphate buffer, pH 7.2, (2) phosphate buffer and 40 % (v/v) trifluoroethanol (TFE) and (3) phosphate buffer and

15   large (100 nm diameter) unilamellar phospholipid vesicles (liposomes) (Table 10). The organic solvent TFE and the liposomes provide a hydrophobic environment intended to mimic the bacterial membrane where the peptides are presumed to adopt an active conformation.

The results indicate that the peptides are primarily unordered in

20   phosphate buffer (a negative minima at around 200 nm) with the exception of MBI 11F4CN, which displays an additional minima at 220 nm (see below). The presence of TFE induces β-turn structure in MBI 11 and MBI 11G4CN, and increases α-helicity in MBI 11F4CN, although most of the peptides remain unordered. In the presence of liposomes, peptides MBI 11CN and MBI 11B7CN, which are unordered in TFE,

25   display β-turn structure (a negative minima at around 230 nm) (Figure 4). Hence, liposomes appear to induce more ordered secondary structure than TFE.

A β-turn is the predominant secondary structure that appears in a hydrophobic environment, suggesting that it is the primary conformation in the active, membrane-associated form. In contrast, MBI 11F4CN displays increased α-helical

30   conformation in the presence of TFE. Peptide MBI 11F4CN is also the most insoluble and hemolytic of the peptides tested, suggesting that α-helical secondary structure may introduce unwanted properties in these analogues.

Additionally CD spectra are recorded for APO-modified peptides (Table 10). The results show that these compounds have significant β-turn secondary structure

35   in phosphate buffer, which is only slightly altered in TFE.

Again, the CD results suggest that a β-turn structure (i.e. membrane-associated) is the preferred active conformation among the indolicidin analogues tested.

Table 10

Peptide	Phosphate buffer		Conformation	TFE		Conformation
	min λ	max λ	in buffer	min λ	max λ	in TFE
MBI 10CN	201	-	Unordered	203	~219	Unordered
MBI 11	199	-	Unordered	202, 227	220	β-turn
MBI 11ACN	199	-	Unordered	203	219	Unordered
MBI 11CN	200	-	Unordered	200	-	Unordered
MBI 11CNY1	200	-	Unordered	200	-	Unordered
MBI 11B1CNW1	201	-	Unordered	201	-	Unordered
MBI 11B4ACN	200	-	Unordered	200	-	Unordered
MBI 11B7CN	200	-	Unordered	204, ~219		Unordered
MBI 11B9ACN	200	-	Unordered	200	-	Unordered
MBI 11B9CN	200	-	Unordered	200	-	Unordered
MBI 11D1CN	200	-	Unordered	204	-	Unordered
MBI 11E1CN	201	-	Unordered	201	-	Unordered
MBI 11E2CN	200	-	Unordered	201	-	Unordered
MBI 11E3CN	202	226	ppII helix	200	-	Unordered
MBI 11F3CN	199	228	ppII helix	202	-	Unordered
MBI 11F4CN	202, 220	-	Unordered	206, 222	-	slight α-helix
MBI 11G4CN	199, 221	-	Unordered	201, 226	215	β-turn
MBI 11G6ACN	200	-	Unordered	199	-	Unordered
MBI	200	-	Unordered	202	221	Unordered

EXAMPLE 9

MEMBRANE PERMEABILIZATION ASSAYS

Liposome dye release

A method for measuring the ability of peptides to permeabilize
5   phospholipid bilayers is described (Parente et al., *Biochemistry*, *29*, 8720, 1990)
Briefly, liposomes of a defined phospholipid composition are prepared in the presence
of a fluorescent dye molecule. In this example, a dye pair consisting of the fluorescent
molecule 8-aminonapthalene-1,3,6-trisulfonic acid (ANTS) and its quencher molecule
p-xylene-bis-pyridinium bromide (DPX) are used. The mixture of free dye molecules,
10   dye free liposomes, and liposomes containing encapsulated ANTS-DPX are separated
by size exclusion chromatography. In the assay, the test peptide is incubated with the
ANTS-DPX containing liposomes and the fluorescence due to ANTS release to the
outside of the liposome is measured over time.

Using this assay, peptide activity, measured by dye release, is shown to
15   be extremely sensitive to the composition of the liposomes at many liposome to peptide
ratios (L/P). Specifically, addition of cholesterol to liposomes composed of egg-
phosphotidylcholine (PC) virtually abolishes membrane permeabilizing activity of MBI
11CN, even at very high lipid to peptide molar ratios (compare with egg PC liposomes
containing no cholesterol). This *in vitro* selectivity may mimic that observed *in vitro*
20   for bacterial cells in the presence of mammalian cells.

In addition, there is a size limitation to the membrane disruption induced
by MBI 11CN. ANTS/DPX can be replaced with fluorescein isothiocyanate-labeled
dextran (FD-4), molecular weight 4,400, in the egg PC liposomes. No increase in FD-4
fluorescence is detected upon incubation with MBI 11CN. These results indicate that
25   MBI 11CN-mediated membrane disruption allows the release of the relatively smaller
ANTS/DPX molecules (~400 Da), but not the bulkier FD-4 molecules.

E. coli ML-35 inner membrane assay

An alternative method for measuring peptide-membrane interaction uses
30   the *E.coli* strain ML-35 (Lehrer et al., *J. Clin. Invest.*, *84:* 553, 1989), which contains a
chromosomal copy of the *lacZ* gene encoding β-galactosidase and is permease
deficient. This strain is used to measure the effect of peptide on the inner membrane
through release of β-galactosidase into the periplasm. Release of β-galactosidase is
measured by spectrophotometrically monitoring the hydrolysis of its substrate *o*-
35   nitrophenol β-D-galactopyranoside (ONPG). The maximum rate of hydrolysis (V_{max}) is
determined for aliquots of cells taken at various growth points.

A preliminary experiment to determine the concentration of peptide
required for maximal activity against mid-log cells, diluted to 4×10^7 CFU/ml, yields a

value of 50 μg/ml, which is used in all subsequent experiments. Cells are grown in two different growth media, Terrific broth (TB) and Luria broth (LB) and equivalent amounts of cells are assayed during their growth cycles. The resulting activity profile of MBI 11B7CN is shown in Figure 5. For cells grown in the enriched TB media,

5   maximum activity occurs at early mid-log (140 min), whereas for cells grown in LB media, the maximum occurs at late mid-log (230 min). Additionally, only in LB, a dip in activity is observed at 140 min. This drop in activity may be related to a transition in metabolism, such as a requirement for utilization of a new energy source due to depletion of the original source, which does not occur in the more enriched TB media.

10   A consequence of a metabolism switch would be changes in the membrane potential.

To test whether membrane potential has an effect on peptide activity, the effect of disrupting the electrochemical gradient using the potassium ionophore valinomycin is examined. Cells pre-incubated with valinomycin are treated with peptide and for MBI 10CN and MBI 11CN ONPG hydrolysis diminished by

15   approximately 50% compared to no pre-incubation with valinomycin. Another cationic peptide that is not sensitive to valinomycin is used as a positive control. Moreover, significant inhibition is observed in a solution containing eith 150 mM NaCl or 5 mM magnesium ions, suggesting involvement of electrostatic interactions in the permeabilizing action of peptides.

20

EXAMPLE 10

PHARMACOLOGY OF PEPTIDE ANALOGUES IN PLASMA AND BLOOD

The *in vitro* lifetime of free peptide analogues in plasma and in blood is determined by measuring the amount of peptide present after set incubation times.

25   Blood is collected from sheep, treated with an anticoagulant (not heparin) and, for plasma preparation, centrifuged to remove cells. Formulated peptide is added to either the plasma fraction or to whole blood and incubated. Following incubation, peptide is identified and quantified directly by reversed phase HPLC. Extraction is not required as the free peptide peak does not overlie any peaks from blood or plasma.

30   A 1 mg/mL solution of MBI 11CN in formulations C1 and D is added to freshly prepared sheep plasma at a final peptide concentration of 100 μg/mL and incubated at 37°C. At various times, aliquots of plasma are removed and analyzed for free peptide by reversed phase HPLC. From each chromatogram, the area of the peak corresponding to free peptide is integrated and plotted against time of incubation. As

35   shown in Figure 6, peptide levels diminish over time. Moreover, when administered in formulation D, up to 50% of the peptide is immediately released from formulation-peptide complex on addition to the blood. The decay curve for free peptide yields an apparent half-life in blood of 90 minutes for both formulation C1 and D. These results

indicate that in sheep's blood MBI 11CN is relatively resistant to plasma peptidases and proteases. New peaks that appeared during incubation may be breakdown products of the peptide.

Peptide levels in plasma *in vivo* are measured after iv or ip 5 administration of 80-100% of the maximum tolerated dose of peptide analogue in either formulation C1 or D. MBI 11CN in formulation C1 is injected intravenously into the tail vein of CD1 ICRBR strain mice. At various times post-injection, mice are anesthetized and blood is drawn by cardiac puncture. Blood from individual mice is centrifuged to separate plasma from cells. Plasma is then analyzed by reversed phase 10 HPLC column. The resulting elution profiles are analyzed for free peptide content by UV absorbance at 280nm, and these data are converted to concentrations in blood based upon a calibrated standard. Each data point represents the average blood level from two mice. In this assay, the detection limit is approximately 1 μg/ml, less than 3% of the dose administered

15   The earliest time point at which peptide can be measured is three minutes following injection, thus, the maximum observed concentration (in μg/ml) is extrapolated back to time zero (Figure 7). The projected initial concentration corresponds well to the expected concentration of between 35 and 45 μg/ml. Decay is rapid, however, and when the curve is fitted to the equation for exponential decay, free 20 circulating peptide is calculated to have a half life of 2.1 minutes. Free circulating peptide was not detectable in the blood of mice that were injected with MBI 11CN in formulation D, suggesting that peptide is not released as quickly from the complex as *in vitro*.

In addition, MBI 11CN is also administered to CD1 ICRBR strain mice 25 by a single ip injection at an efficacious dose level of 40 mg/kg. Peptide is administered in both formulations C1 and D to determine if peptide complexation has any effect on blood levels. At various times post injection, mice are anesthetized and blood is drawn by cardiac puncture. Blood is collected and analyzed as for the iv injection.

30   MBI 11CN administered by this route demonstrated a quite different pharmacologic profile (Figure 8). In formulation C1, peptide entered the blood stream quickly, with a peak concentration of nearly 5 μg/ml after 15 minutes, which declined to non-detectable levels after 60 minutes. In contrast, peptide in formulation D is present at a level above 2 μg/ml for approximately two hours. Therefore, formulation 35 affects entry into, and maintenance of levels of peptide in the blood.

EXAMPLE 11

TOXICITY OF PEPTIDES *IN VIVO*

The acute, single dose toxicity of various indolicidin analogues is tested in Swiss CD1 mice using various routes of administration. In order to determine the inherent toxicities of the peptide analogues in the absence of any formulation/delivery vehicle effects, the peptides are all administered in isotonic saline with the final pH between 6 and 7.

Intraperitoneal route. Groups of 6 mice are injected with peptide doses of between 80 and 5 mg/kg in 500 μl dose volumes. After peptide administration, the mice are observed for a period of 5 days, at which time the dose causing 50% mortality (LD_{50}), the dose causing 90-100% mortality (LD_{90-100}) and maximum tolerated dose (MTD) levels are determined. The LD_{50} values are calculated using the method of Reed and Muench (*J. of Amer. Hyg.* 27: 493-497, 1938). The results presented in Table 11 show that the LD_{50} values for MBI 11CN and analogues range from 21 to 52 mg/kg.

Table 11

Peptide	LD_{50}	LD_{90-100}	MTD
MBI 11CN	34 mg/kg	40 mg/kg	20 mg/kg
MBI 11B7CN	52 mg/kg	>80 mg/kg	30 mg/kg
MBI 11E3CN	21 mg/kg	40 mg/kg	<20 mg/kg
MBI 11F3CN	52 mg/kg	80 mg/kg	20 mg/kg

Intravenous route. Groups of 6 mice are injected with peptide doses of 20, 16, 12, 8, 4 and 0 mg/kg in 100 μl volumes (4 ml/kg). After administration, the mice are observed for a period of 5 days, at which time the LD_{50}, LD_{90-100} and MTD levels are determined. The results from the IV toxicity testing of MBI 11CN and three analogues are shown in Table 11. The LD_{50}, LD_{90-100} and MTD values range from 5.8 to 15 mg/kg, 8 to 20 mg/kg and <4 to 12 mg/kg respectively.

Table 12

Peptide	LD_{50}	LD_{90-100}	MTD
MBI 11CN HCl	5.8 mg/kg	8.0 mg/kg	<4 mg/kg
MBI 11B7CN HCl	7.5 mg/kg	16 mg/kg	4 mg/kg
MBI 11F3CN HCl	10 mg/kg	12 mg/kg	8 mg/kg
MBI 11F4CN HCl	15 mg/kg	20 mg/kg	12 mg/kg

Subcutaneous route. The toxicity of MBI 11CN is also determined after subcutaneous (SC) administration. For SC toxicity testing, groups of 6 mice are injected with peptide doses of 128, 96, 64, 32 and 0 mg/kg in 300 μL dose volumes (12 mL/kg). After administration, the mice are observed for a period of 5 days. None of the animals died at any of the dose levels within the 5 day observation period. Therefore, the LD_{50}, LD_{90-100} and MTD are all taken to be greater than 128 mg/kg. Mice receiving higher dose levels showed symptoms similar to those seen after IV injection suggesting that peptide entered the systemic circulation. These symptoms are reversible, disappearing in all mice by the second day of observations.

The single dose toxicity of MBI 10CN and MBI 11CN in different formulations is also examined in outbred ICR mice (Table 13). Intraperitoneal injection (groups of 2 mice) of MBI 10CN in formulation D show no toxicity up to 29 mg/kg and under the same conditions MBI 11CN show no toxicity up to 40 mg/kg.

Intravenous injection (groups of 10 mice) of MBI 10CN in formulation D show a maximum tolerated dose (MTD) of 5.6 mg/kg (Table 13). Injection of 11 mg/kg gave 40% toxicity and 22 mg/kg result in 100% toxicity. Intravenous injection of MBI 11CN in formulation C (lyophilized) show a MTD of 3.0 mg/kg. Injection at 6.1 mg/kg result in 10% toxicity and at 12 mg/kg 100% toxicity.

Table 13

Peptide	Route	# Animals	Formulation	MTD (mg/kg)
MBI 10CN	ip	2	formulation D	>29
MBI 11CN	ip	2	formulation D	>40
MBI 10CN	iv	10	formulation D	5.6
MBI 11CN	iv	10	formulation C (lyophilized)	3.0

These results are obtained using peptide/buffer solutions that are lyophilized after preparation and reconstituted with water. If the peptide solution is not lyophilized before injection, but used immediately after preparation, an increase in toxicity is seen, and the maximum tolerated dose can decrease by up to four-fold. For example, an intravenous injection of MBI 11CN as a non-lyophilized solution, formulation C1, at 1.5 mg/kg results in 20% toxicity and at 3.0 mg/kg gave 100% toxicity. HPLC analyses of the non-lyophilized and lyophilized formulations indicate

that the MBI 11CN forms a complex with polysorbate, and this complexation of the peptide reduces its toxicity in mice.

In addition, mice are multiply injected by an intravenous route with MBI 11CN (Table 14). In one representative experiment, peptide administered in 10 injections of 0.84 mg/kg at 5 minute intervals is not lethal. However, two injections of peptide at 4.1 mg/kg administered with a 10 minute interval results in 60% mortality.

Table 14

Peptide	Route	Formulation	Dose Level*	# Injections	Time Interval	Result
MBI 11CN	iv	formulation D	0.84	10	5 min	no mortality
MBI 11CN	iv	formulation D	4.1	2	10 min	66% mortality

* (mg/kg)

To assess the impact of dosing mice with peptide analogue, a series of histopathology investigations can be carried out. Groups of mice are administered analogue at dose levels that are either at, or below the MTD, or above the MTD, a lethal dose. Multiple injections may be used to mimic possible treatment regimes. Groups of control mice are not injected or injected with buffer only.

Following injection, mice are sacrificed at specified times and their organs immediately placed in a 10% balanced formalin solution. Mice that die as a result of the toxic effects of the analogue also have their organs preserved immediately. Tissue samples are taken and prepared as stained micro-sections on slides which are then examined microscopically. Damage to tissues is assessed and this information can be used to develop improved analogues, improved methods of administration or improved dosing regimes.

EXAMPLE 12

IN VIVO EFFICACY OF PEPTIDES

Analogues are tested for their ability to rescue mice from lethal bacterial infections. The animal model used is an intraperitoneal (ip) inoculation of mice with 10^6-10^8 Gram-positive organisms with subsequent administration of peptide. The three pathogens investigated, methicillin-sensitive *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), or *S. epidermidis* are injected ip into mice. For untreated mice, death occurs within 12-18 hours with MSSA and *S. epidermis* and within 6-10 hours with MRSA.

Peptide is administered by two routes, intraperitoneally, at one hour post-infection, or intravenously, with single or multiple doses given at various times pre- and post-infection.

MSSA infection. In a typical protocol, groups of 10 mice are infected
5 intraperitoneally with a LD_{90-100} dose (5.2 x 10^6 CFU/mouse) of MSSA (Smith, ATCC # 19640) injected in brain-heart infusion containing 5% mucin. This strain of *S. aureus* is not resistant to any common antibiotics. At 60 minutes post-infection, MBI 10CN or MBI 11CN, in formulation D, is injected intraperitoneally at the stated dose levels. An injection of formulation alone serves as a negative control and administration of
10 ampicillin serves as a positive control. The survival of the mice is monitored at 1, 2, 3 and 4 hrs post-infection and twice daily thereafter for a total of 8 days.

As shown in Figure 9, MBI 10CN is maximally active against MSSA (70-80% survival) at doses of 14.5 to 38.0 mg/kg, although 100% survival is not achieved. Below 14.5 mg/kg, there is clear dose-dependent survival. At these lower
15 dose levels, there appears to be an animal-dependent threshold, as the mice either die by day 2 or survive for the full eight day period. As seen in Figure 10, MBI 11CN, on the other hand, rescued 100% of the mice from MSSA infection at a dose level of 35.7 mg/kg, and was therefore as effective as ampicillin. There was little or no activity at any of the lower dose levels, which indicates that a minimum bloodstream peptide
20 level must be achieved during the time that bacteria are a danger to the host.

As shown above, blood levels of MBI 11CN can be sustained at a level of greater than 2 μg/ml for a two hour period inferring that this is higher than the minimum level.

Additionally, eight variants based on the sequence of MBI 11CN are
25 tested against MSSA using the experimental system described above. Peptides prepared in formulation D are administered at dose levels ranging from 12 to 24 mg/kg and the survival of the infected mice is monitored for eight days (Figures 11-19). The percentage survival at the end of the observation period for each variant is summarized in Table 15. As shown in the table, several of the variants showed efficacy greater than
30 or equal to MBI 11CN under these conditions.

Table 15

% Survival	24 mg/kg	18 mg/kg	12 mg/kg
100			
90	11B1CN, 11F3CN		
80			
70		11E3CN	
60	11B7CN		
50	11CN		
40	11G2CN		
30		11B1CN	
20	11G4CN		
10		11CN, 11B7CN, 11B8CN, 11F3CN	11G2CN
0	11A1CN	11A1CN, 11G2CN, 11G4CN	11CN, 11A1CN, 11B1CN, 11B7CN, 11B8CN, 11F3CN, 11G4CN

S. epidermidis infection. Peptide analogues generally have lower MIC values against *S. epidermidis in vitro*, therefore, lower blood peptide levels might be more effective against infection.

In a typical protocol, groups of 10 mice are injected intraperitoneally with an LD_{90-100} dose (2.0×10^8 CFU/mouse) of *S. epidermidis* (ATCC # 12228) in brain-heart infusion broth containing 5% mucin. This strain of *S. epidermidis* is 90% lethal after 5 days. At 15 mins and 60 mins post-infection, various doses of MBI 11CN in formulation D are injected intravenously via the tail vein. An injection of formulation only serves as the negative control and injection of gentamicin serves as the positive control; both are injected at 60 minutes post-infection. The survival of the mice is monitored at 1, 2, 3, 4, 6 and 8 hrs post-infection and twice daily thereafter for a total of 8 days.

As shown in Figures 20A and 20B, MBI 11CN prolongs the survival of the mice. Efficacy is observed at all three dose levels with treatment 15 minutes post-infection, however, there is less activity at 30 minutes post-infection and no significant effect at 60 minutes post-infection. Time of administration appears to be important in this model system, with a single injection of 6.1 mg/kg 15 minutes post-infection giving the best survival rate.

MRSA infection. MRSA infection, while lethal in a short period of time, requires a much higher bacterial load than MSSA. In a typical protocol, groups of 10 mice are injected intraperitoneally with a $LD_{90\text{-}100}$ dose (4.2×10^7 CFU/mouse) of MRSA (ATCC # 33591) in brain-heart infusion containing 5% mucin. The treatment
5   protocols are as follows, with the treatment times relative to the time of infection:

- 0 mg/kg   Formulation D alone (negative control), injected at 0 mins

- 5 mg/kg   Three 5.5 mg/kg injections at -5, +55, and +115 mins

- 1 mg/kg (2 hr)   Five 1.1 mg/kg injections at -5, +55, +115, +175 and +235 mins.

- 1 mg/kg (20 min)   Five 1.1 mg/kg injections at -10, -5, 0, +5, and +10 mins

10   • Vancomycin   (positive control) injected at 0 mins

MBI 11CN is injected intravenously in the tail vein in formulation D. Survival of mice is recorded at 1, 2, 3, 4, 6, 8, 10, 12, 20, 24 and 30 hrs post-infection and twice daily thereafter for a total of 8 days. There was no change in the number of surviving mice after 24 hrs (Figure 21).
15   The 1 mg/kg (20 min) treatment protocol, with injections 5 minutes apart centered on the infection time, delayed the death of the mice to a significant extent with one survivor remaining at the end of the study. The results presented in Table 16 suggest that a sufficiently high level of MBI 11CN maintained over a longer time period would increase the number of mice surviving. The 5 mg/kg and 1 mg/kg (2 hr) results, where there is no improvement in survivability over the negative control,
20   indicates that injections 1 hour apart, even at a higher level, are not effective against MRSA.

Table 16

25

Time of Observation (Hours post-infection)	Percentage of Animals Surviving	
	No Treatment	Treatment
6	50%	70%
8	0	40%
10	0	30%
12	0	20%

EXAMPLE 13

CYTOTOXICITY OF CATIONIC PEPTIDES IN NORMAL AND IN TUMOR CELLS

Studies were performed to determine whether there is a difference in the sensitivity to cytotoxic agents in normal cells and tumor cells. In these experiments, cells were exposed to peptide for four hours at 37°C. Tissue culture supernatant was then collected and measured for lactate dehydrogenase activity. Cell survival was determined by the fraction of lactate dehydrogenase released into the supernatant. The results are shown in Figure 22, which illustrates sensitivity of normal human PBL and two hematopoietic tumor cell lines to five peptides. This study examined the "11 series peptides," which are peptides that are variants of the cationic peptide designated, 11CN. Additional results of this study indicated that the toxicity of the 11 series peptides towards peripheral blood lymphocytes (PBL) was very similar to the cytotoxicity observed towards hematopoietic cells (K562 and DOHH-2), and generally higher than the cytotoxicity against solid tumor cells (H460 and MCF-7). A "20 series peptide" (*i.e.*, a peptide of the group MBI 20 - MBI 29) was also examined. MBI 29 was found to be relatively less toxic towards PBL than towards the hematopoietic tumor cells.

In further studies, the 11 series peptides and 20 series peptides were found to be less toxic towards HUVEC cells than towards tumor cells or towards PBL cells. This difference was greatest with 11CN and 11E3CN. The HUVEC cells can be maintained at confluence for several days. Exposing the cells to peptide either while actively growing or while at confluence did not greatly influence sensitivity to peptides.

Comparative cytotoxicity tests were also performed with chemotherapeutic agents on normal cells. Two agents, doxorubicin and vincristine sulfate were obtained from the B.C. Cancer Agency. In this study, cells were exposed to agents for three days. The surviving cell fraction was determined by the MTT assay. Figure 23 shows the concentration of agent yielding 50% cell survival. The results indicate that the chemotherapeutic agents do not have a high selectivity for normal cells over tumor cells in the *in vitro* assay system. For instance, peripheral blood lymphocytes (A) and MCF-7 cells (F) have similar sensitivities to both doxorubicin and vincristine sulphate.

The activities of APO-modified cationic peptides was also examined. As an illustration of APO-modified cationic peptides, peptides were modified with TWEEN 80, as described above. In this study, cells were exposed to peptide for four hours at 37°C. Tissue culture supernatant was collected and measured for lactate dehydrogenase activity. Cell survival was determined by the fraction of lactate dehydrogenase released into the supernatant. The results for two peptides, 11CN and 11E3CN, compared with their modified counterparts (11CN-T and 11E3CN-T), are shown in Figures 24 and 25, respectively. With one exception, both normal cells and

tumor cells were more sensitive to the modified formulations. H460 cells, however, were more sensitive to 11B7CN than to 11B7CN-T.

5

10

Preliminary information suggested that multidrug resistant (MDR) cells do not display resistance to MBI peptides. Data were collected for both wild-type MCF-7 cells and MCF-7 cells which express the multi-drug resistant phenotype, MCF-7ADR and for P388 cells and their MDR counterpart P388 ADR cells. In this study, cells were exposed to peptide for four hours at 37°C. Tissue culture supernatant was collected and measured for lactate dehydrogenase activity. Cell survival was determined by the fraction of lactate dehydrogenase released into the supernatant. As shown in Figure 26, MCF-7 ADR cells may have slightly elevated sensitivity in comparison to their wild-type parent cells. However, P388 wild type and MDR cells show very similar sensitivity to MBI peptides, as shown in Figure 27.

15

In sum, all 11 series MBI peptides (except 11A3CN, which was not lytic to cells within the tested concentration range) and MBI 28 were cytotoxic to tumor cell lines at lower concentrations than were required for HUVEC cells. One peptide, MBI 29, showed significant hemolytic activity. Collectively, the data indicate that the peptides are useful as tumor-selective agents. In addition, the sensitivity of cells to MBI peptides was not affected by expression of the MDR phenotype.

20

EXAMPLE 14
THERAPY WITH CATIONIC PEPTIDES AND CONVENTIONAL ANTINEOPLASTIC AGENTS

25

30

To identify a possible augmentation effect by MBI peptides, peptides were tested in combination with conventional chemotherapeutic agents. Since these assays require longer incubations in serum containing media, it was necessary to determine whether the effect of the peptide on the cells was not hindered by the presence of serum. An LDH assay was performed with either 11E3CN and 11CN in the absence of serum or in the presence of serum. Serum did not reduce the cytotoxicity of either peptide. Moreover, incubating 11E3CN at 37°C in media for one hour prior to its addition to cells did not reduce its cytoxicity. The MTT assays and the LDH assays yielded similar cytotoxicity values. That is, the IC_{50} measured by a MTT assay and the LC_{50} measured with the LDH assay were similar, as illustrated by Figure 28, in which cells had been incubated for three days in the absence or presence of cationic peptide.

35

Studies indicate that MDR cells show increased sensitivity to doxorubicin in the presence of very low concentrations of cationic peptides. In one study, P388 ADR cells were seeded at 4000 cells per well, and medium was changed to 20% serum just prior to addition of agent. Peptide and doxorubicin were diluted in RPMI without serum, thus the final concentration of serum was 10%. Doxorubicin was added, followed one-half hour later by the addition of 11E3CN. In this experiment, the

IC_{50} of 11E3CN was determined to be 25 μM. As shown in Figure 29, the combination of the cationic peptide and doxorubicin produced a synergistic effect.

A synergistic effect was also observed in a study with multidrug resistant MCF-7 cells. In this experiment, MCF-7ADR cells were seeded at 2000 cells per well in media containing 10% fetal bovine serum. Peptide and doxorubicin were diluted in RPMI containing 10% fetal bovine serum. Doxorubicin was added to the cultures, followed one-half hour later by the addition of 11CN. The results are shown in Figure 30. The IC_{50} of 11CN in this study was determined to be 15 μM.

In sum, a synergistic effect was observed with both P388 ADR cells (Figure 29) and MCF-7 ADR cells (Figure 30) with two peptides, 11CN and 11E3CN. The cytotoxic reaction of MDR cells to doxorubicin was observed at concentrations of peptide below which any reduction of cell viability was observed for the peptide alone. For instance, P388 cells were sensitized to doxorubicin at 4 μM 11E3CN, which represents one-fifth of the IC_{50} concentration, and is a concentration of peptide at which no inhibition of cell growth was observed with peptide alone. The IC_{50} of doxorubicin decreased from 5 μM (doxorubicin given alone) to 0.25 μM (doxorubicin given with 11E3CN).

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following claims.

CLAIMS

We claim:

1.   A pharmaceutical composition, comprising at least one cationic peptide and a pharmaceutically acceptable carrier, wherein said cationic peptide is an APO-modified cationic peptide or a cationic peptide selected from the group consisting of MBI 11A9CN (I L R W P W W P W W P W R R K), MBI 11A10CN (W W R W P W W P W R R K), MBI 11B7CN (I L R W P W W P W R R K), MBI 11B19CN (I L R W P W R R W P W R R K), MBI 11B20CN (I L R W P W W P W R R K I L M R W P W W P W R R K M A A), MBI 11D19CN (C L R W P W W P W R R K), MBI 11E3CN (i L K K W P W W P W R R k), MBI 11F4CN (I L R W V W W V W R R K), MBI 11F5CN (I L R R W V W W V W R R K), MBI 11F6CN (I L R W W V W W V W W R R K), MBI 11G25CN (L R W W W P W R R K), MBI 11G26CN (L R W P W W P W), MBI 11G28CN (R W W W P W R R K), MBI 11J01CN (R R I W K P K W R L P K R), MBI 11J02CN (W R W W K P K W R W P K W), and MBI 29 (K W K S F I K K L T T A V K K V L T T G L P A L I S).

2.   The pharmaceutical composition of claim 1, further comprising at least one antineoplastic drug.

3.   The pharmaceutical composition of claim 1, wherein said composition comprises at least one modified cationic peptide.

4.   The pharmaceutical composition of claim 3, wherein said modified cationic peptide is a conjugate comprising an activated polyoxyalkylene and a lipophilic moiety.

5.   The pharmaceutical composition of claim 4, wherein said polyoxyalkylene is polyoxyalkylene glycol.

6.   The pharmaceutical composition of claim 4, wherein said lipophilic moiety is a fatty acid.

7.   The pharmaceutical composition of claim 4, wherein said polyoxyalkylene is polyoxyalkylene glycol and said lipophilic moiety is a fatty acid.

8.   The pharmaceutical composition of claim 7, wherein said conjugate further comprises sorbitan linking said polyoxyalkylene glycol and fatty acid.

9.   The pharmaceutical composition of claim 8, wherein the said conjugate is polysorbate.

10.   The pharmaceutical composition of claim 5, wherein said polyoxyalkylene glycol is polyoxyethylene.

11.   The pharmaceutical composition of claim 3, wherein said modified cationic peptide is a conjugate of a cationic peptide and Tween 80.

12.   The pharmaceutical composition of claim 2, wherein said composition comprises at least one antineoplastic agent selected from the group consisting of nitrogen mustard, alkyl sulfonate, nitrosourea, triazene, folic acid analog, pyrimidine analog, purine analog, epipodophyllotoxin, and platinum coordination complex.

13.   A method of treating a subject having a tumor, comprising the step of administering a pharmaceutical composition that comprises at least one cationic peptide and a pharmaceutically acceptable carrier, wherein said cationic peptide is an APO-modified cationic peptide or a cationic peptide selected from the group consisting of MBI 11A9CN (I L R W P W W P W W P W R R K), MBI 11A10CN (W W R W P W W P W R R K), MBI 11B7CN (I L R W P W W P W R R K), MBI 11B19CN (I L R W P W R R W P W R R K), MBI 11B20CN (I L R W P W W P W R R K I L M R W P W W P W R R K M A A), MBI 11D19CN (C L R W P W W P W R R K), MBI 11E3CN (i L K K W P W W P W R R k), MBI 11F4CN (I L R W V W W V W R R K), MBI 11F5CN (I L R R W V W W V W R R K), MBI 11F6CN (I L R W W V W W V W W R R K), MBI 11G25CN (L R W W W P W R R K), MBI 11G26CN (L R W P W W P W), MBI 11G28CN (R W W W P W R R K), MBI 11J01CN (R R I W K P K W R L P K R), MBI 11J02CN (W R W W K P K W R W P K W), and MBI 29 (K W K S F I K K L T T A V K K V L T T G L P A L I S).

14.   The method of claim 13, wherein said tumor is selected from the group consisting of lymphoma, leukemia, multiple myeloma, breast tumor, lung tumor, ovarian tumor, cervical tumor, uterine tumor, skin tumor, prostate tumor, liver tumor, and colon tumor.

15.   The method of claim 13, wherein said tumor comprises multidrug resistant cells.

16.   The method of claim 13, wherein said pharmaceutical composition is administered by a mode selected from the group consisting of intravenous administration, intraperitoneal administration, intramuscular administration, subcutaneous administration, and intralesional administration.

17.   The method of claim 13, wherein said pharmaceutical composition comprises at least one modified cationic peptide.

18.   The method of claim 17, wherein said modified cationic peptide is a conjugate comprising an activated polyoxyalkylene and a lipophilic moiety.

19.   The method of claim 18, wherein said polyoxyalkylene is polyoxyalkylene glycol.

20.   The method of claim 18, wherein said lipophilic moiety is a fatty acid.

21.   The method of claim 18, wherein said polyoxyalkylene is polyoxyalkylene glycol and said lipophilic moiety is a fatty acid.

22.   The method of claim 21, wherein said conjugate further comprises sorbitan linking said polyoxyalkylene glycol and fatty acid.

23.   The method of claim 22, wherein the said conjugate is polysorbate.

24.   The method of claim 19, wherein said polyoxyalkylene glycol is polyoxyethylene.

25.   The method of claim 17, wherein said modified cationic peptide is a conjugate of a cationic peptide and Tween 80.

26.   The method of claim 13, further comprising the step of administering one antineoplastic agent to said subject, wherein said antineoplastic agent is selected from the

group consisting of nitrogen mustard, alkyl sulfonate, nitrosourea, triazene, folic acid analog, pyrimidine analog, purine analog, epipodophyllotoxin, and platinum coordination complex.

27.   The method of claim 26, wherein said antineoplastic agent is administered prior to the administration of said pharmaceutical composition comprising least one cationic peptide.

28.   The method of claim 26, wherein said antineoplastic agent is administered concomitantly with the administration of said pharmaceutical composition comprising least one cationic peptide.

29.   The method of claim 28, wherein said pharmaceutical composition comprises at least one antineoplastic agent.

30.   The method of claim 26, wherein said antineoplastic agent is administered after the administration of said pharmaceutical composition comprising least one cationic peptide.

Fig. 2B

4/24

Fig. 3A

Fig. 3B

Fig. 3C

Fig. 4A

Fig. 4B

Fig. 5A

Fig. 5B

Fig. 6

Fig. 7

Fig. 8

Fig. 9

9/24
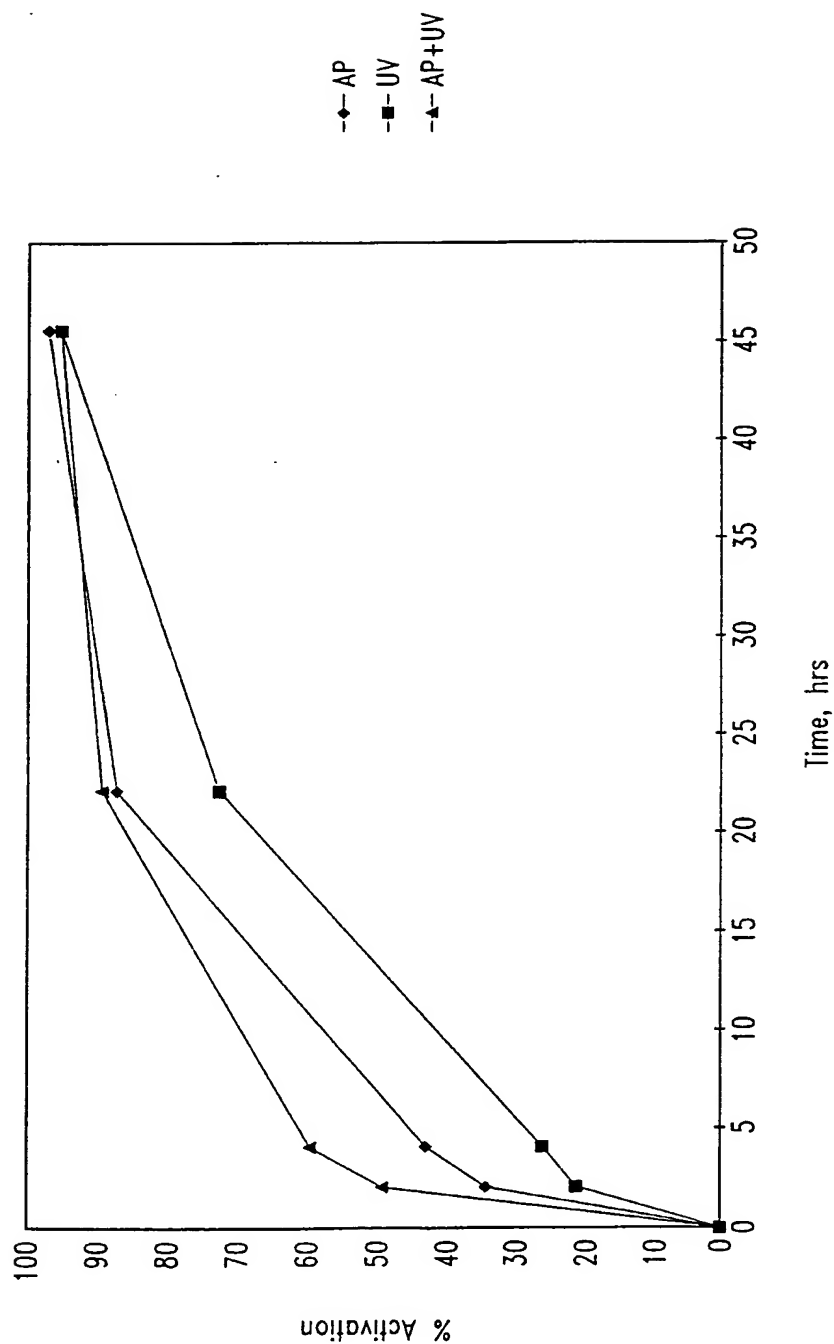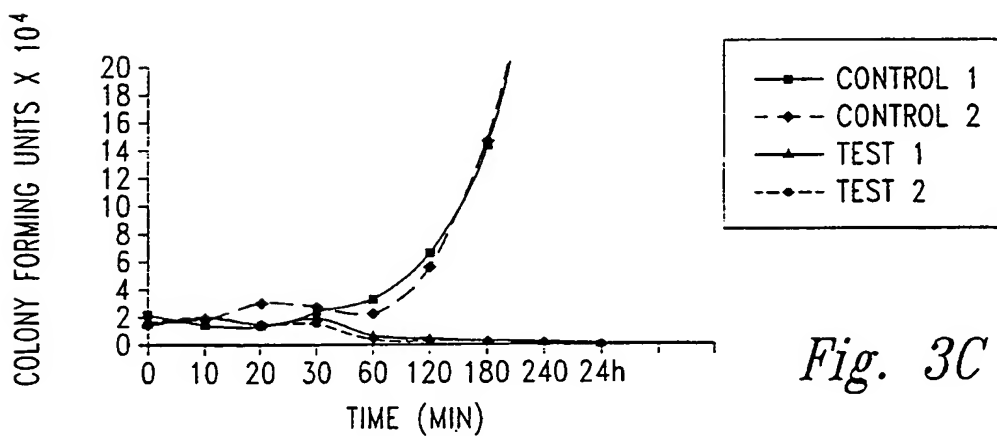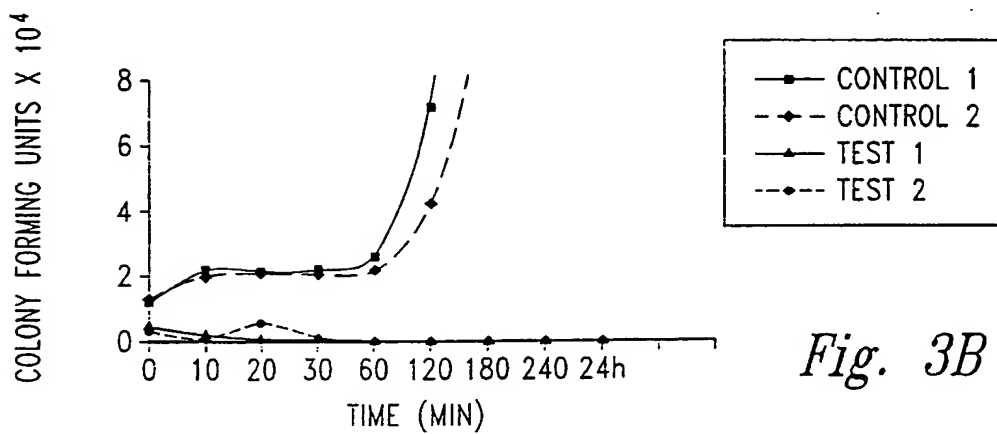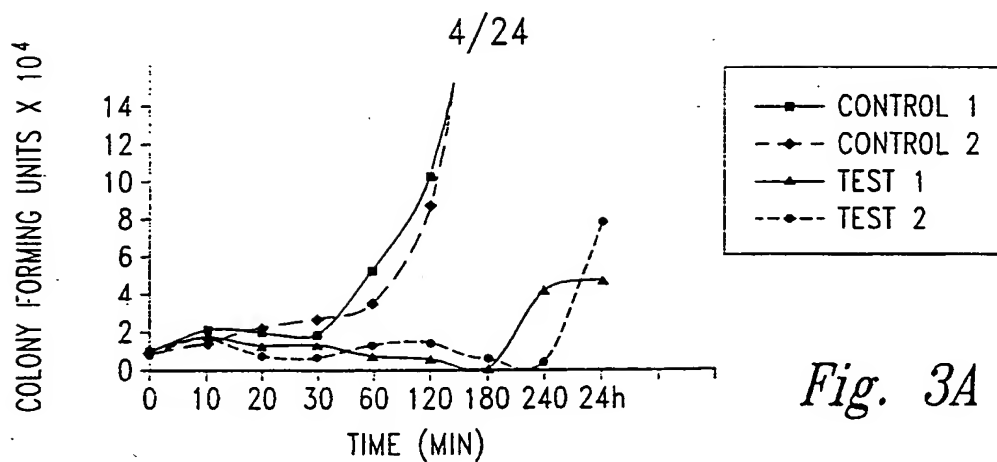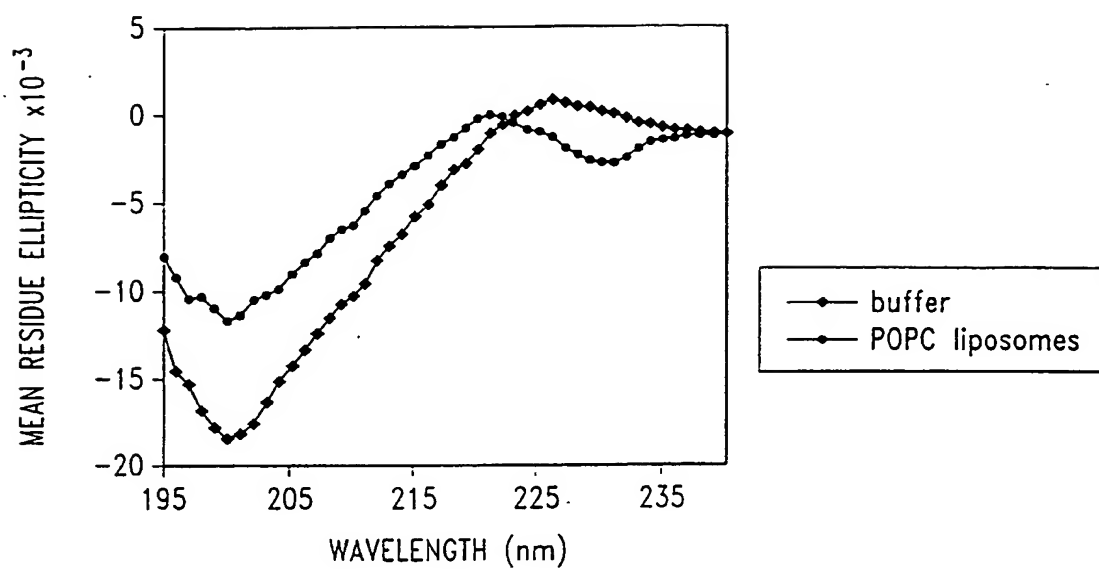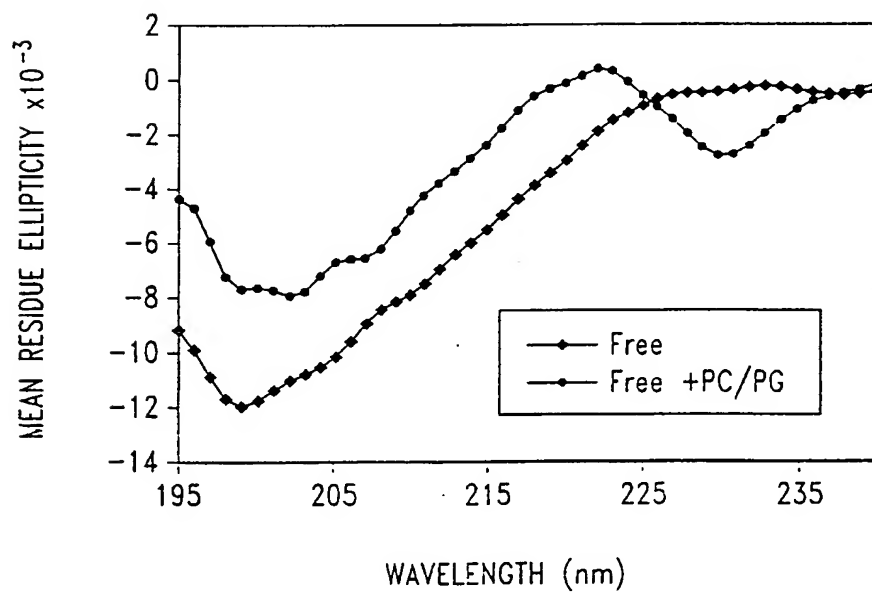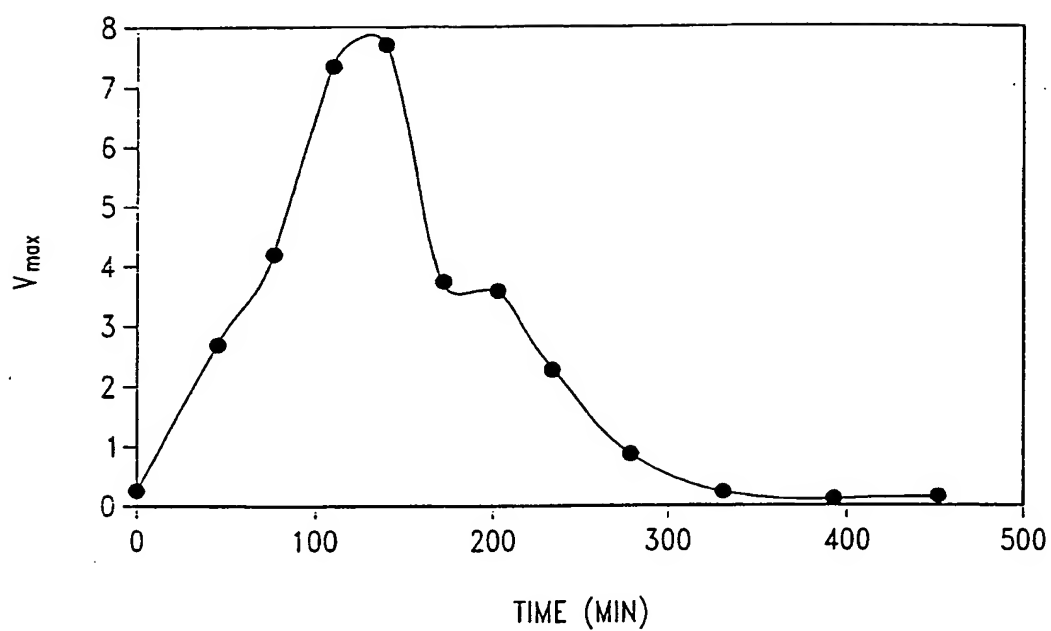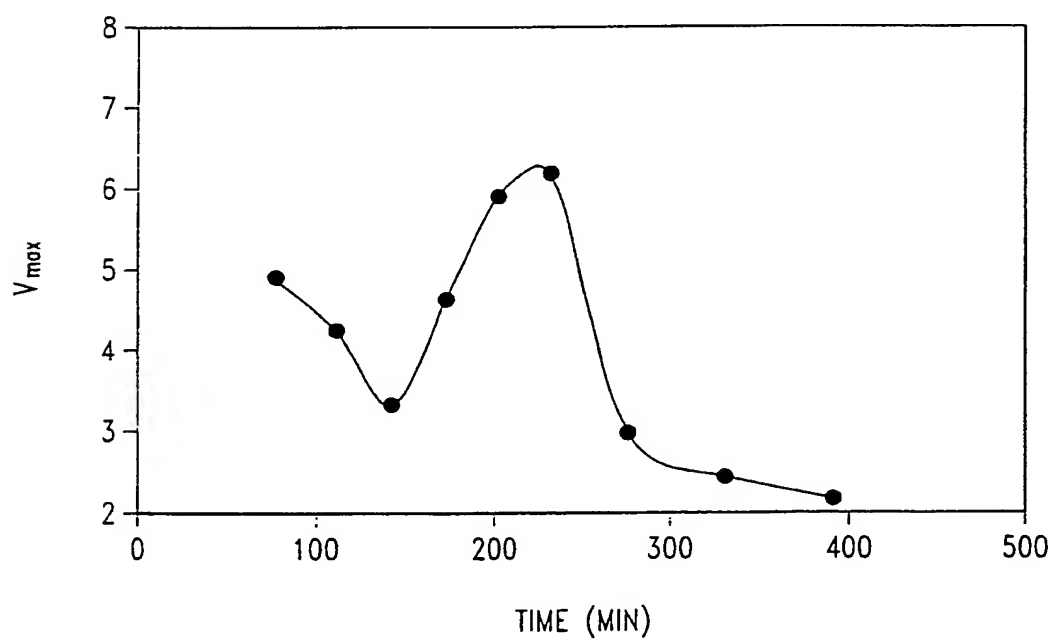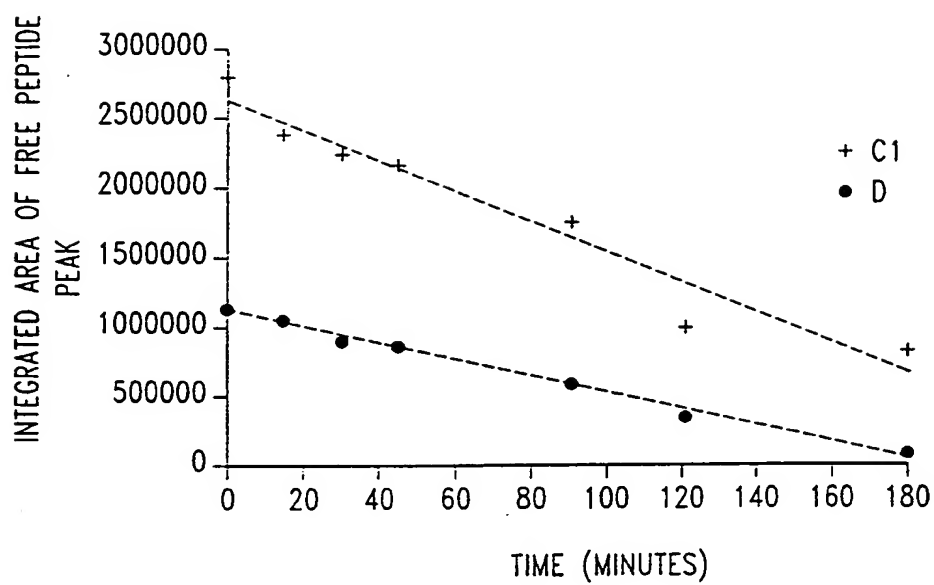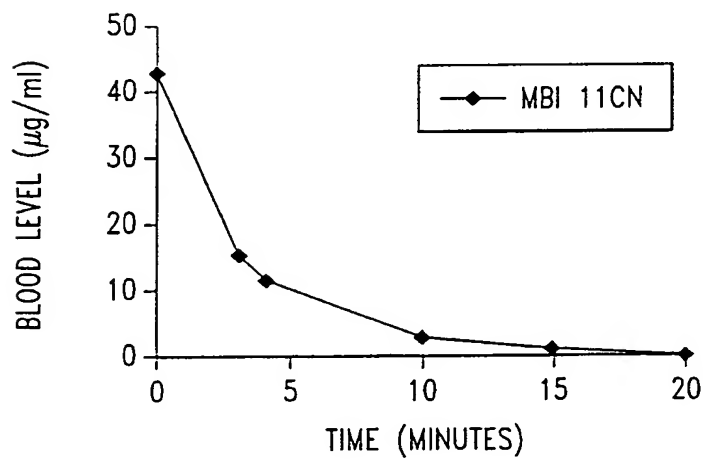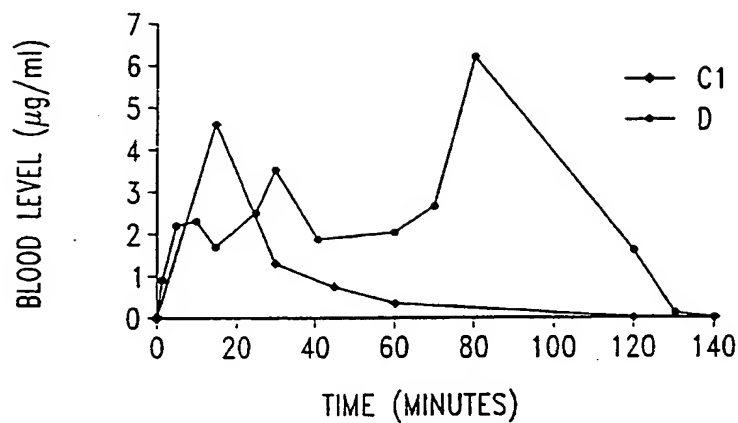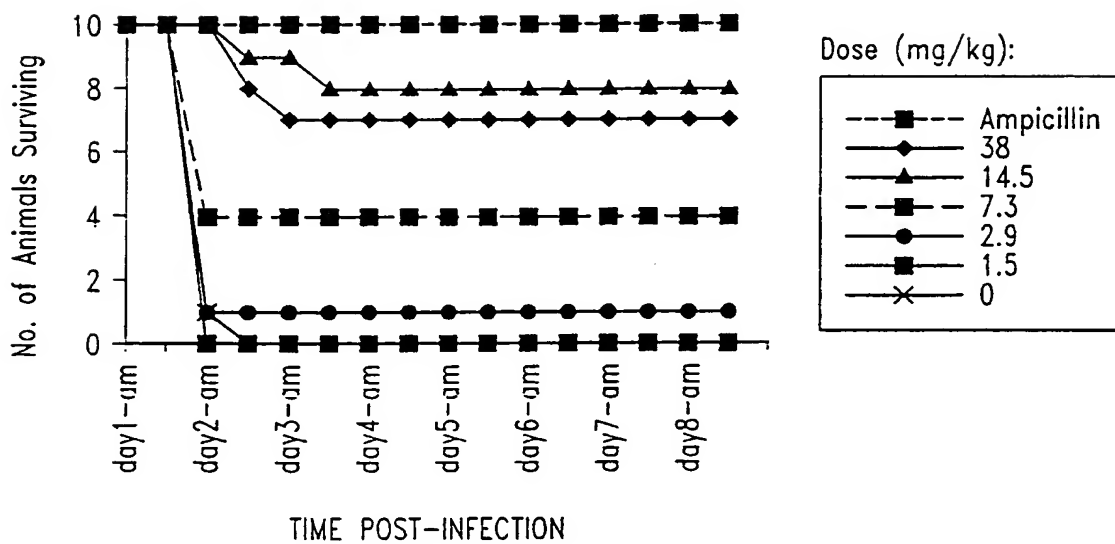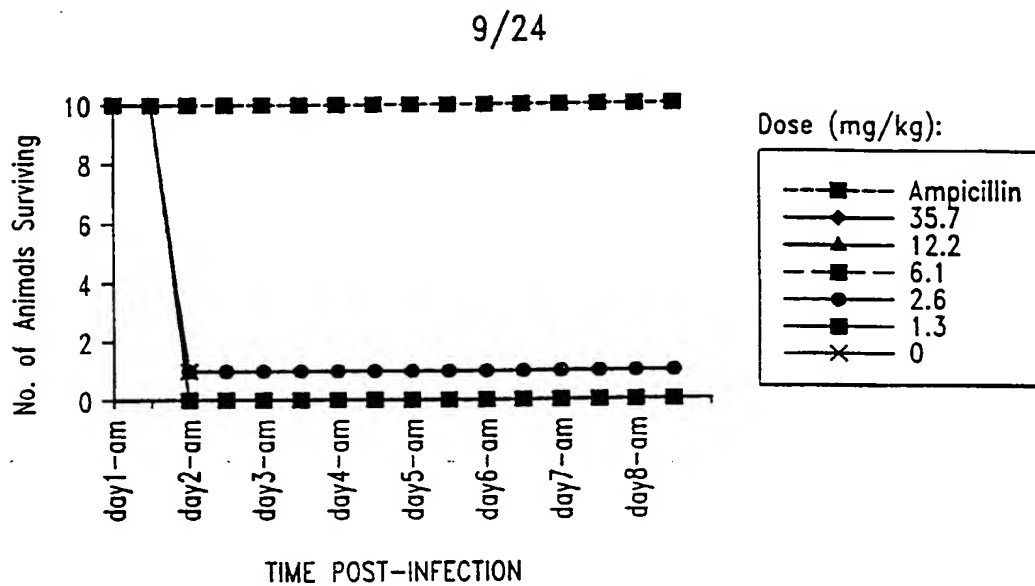
Fig. 10
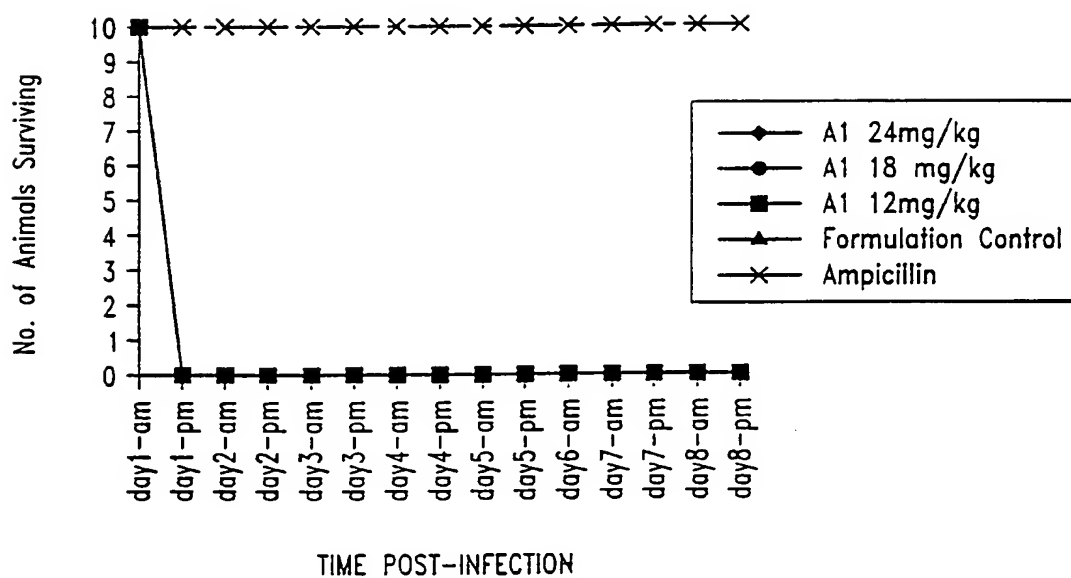
Fig. 11
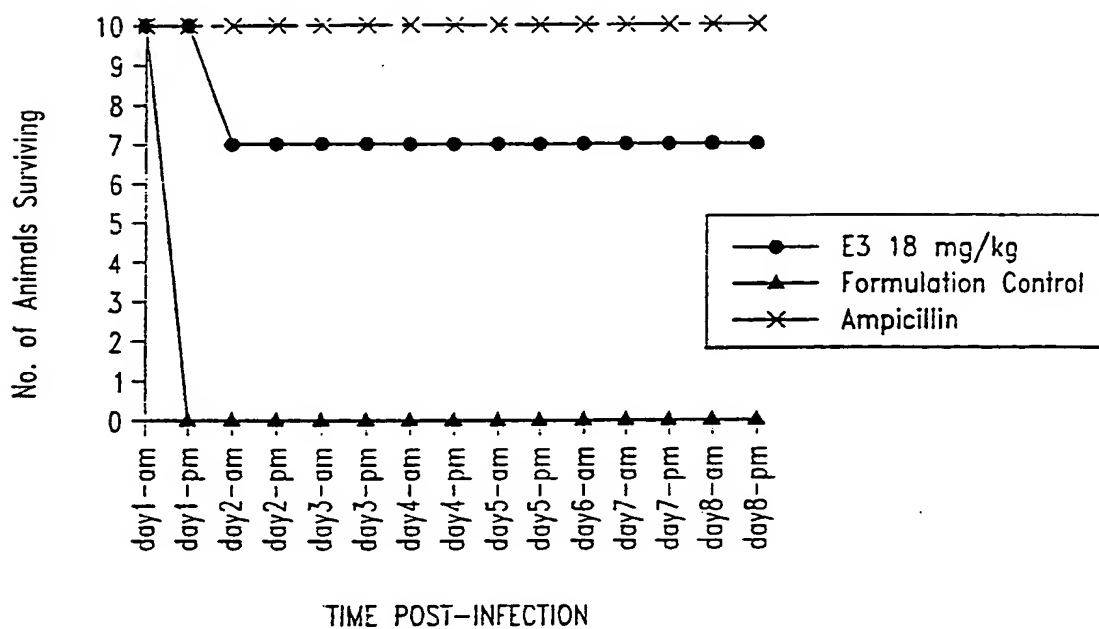
Fig. 12
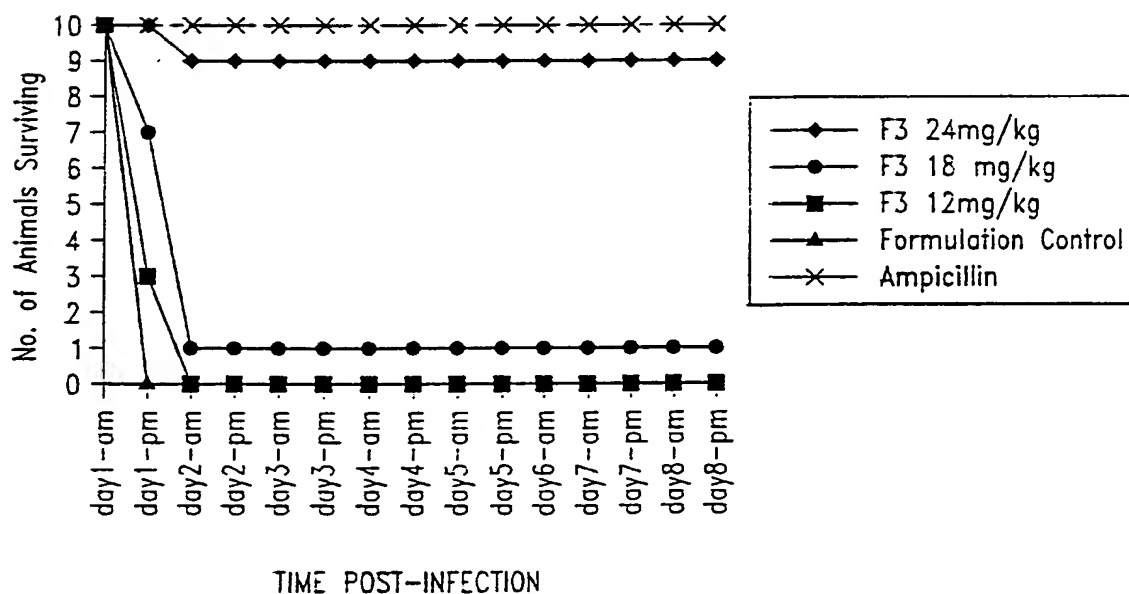
Fig. 13
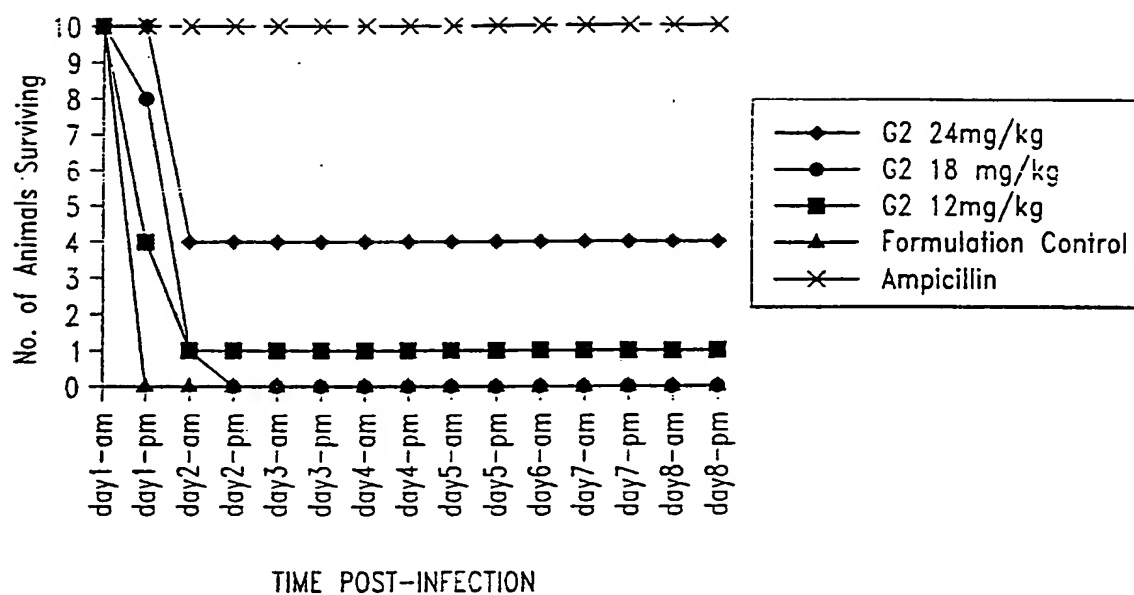
Fig. 14
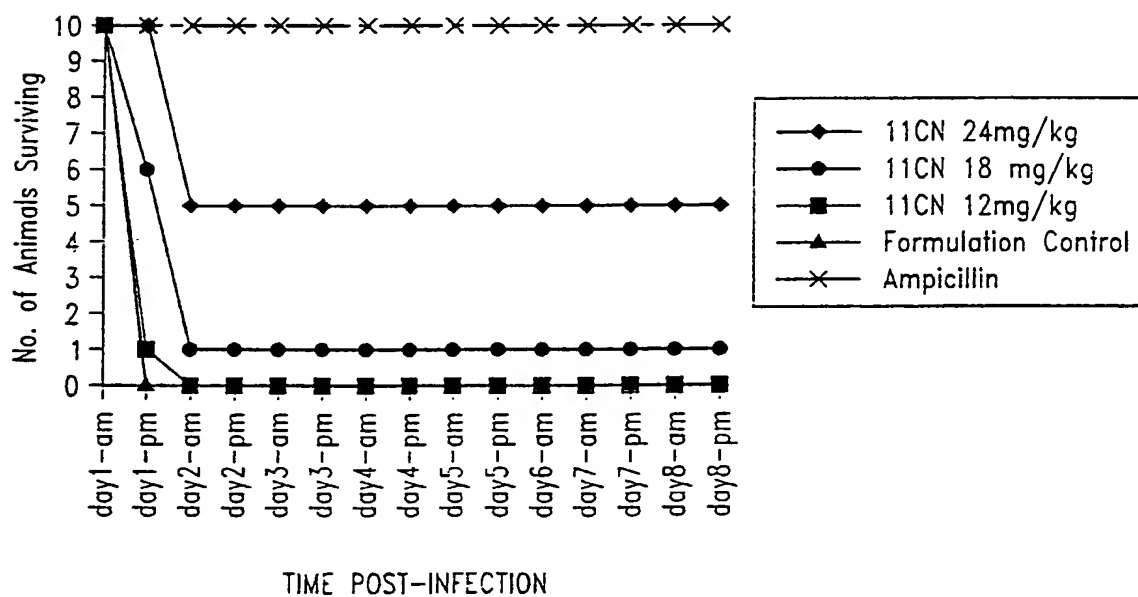
Fig. 15
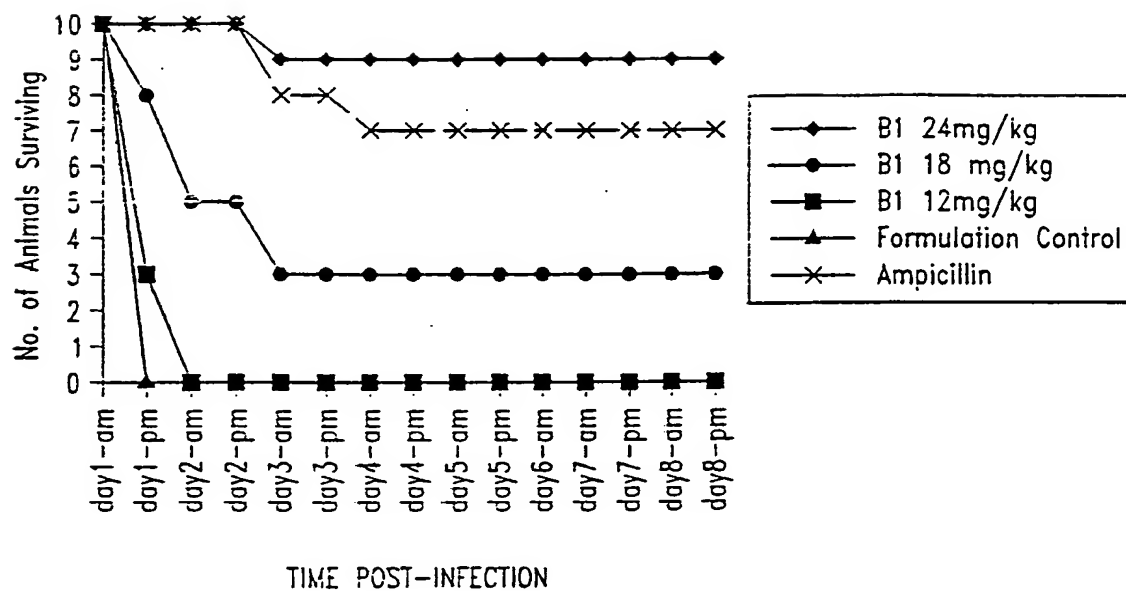
Fig. 16
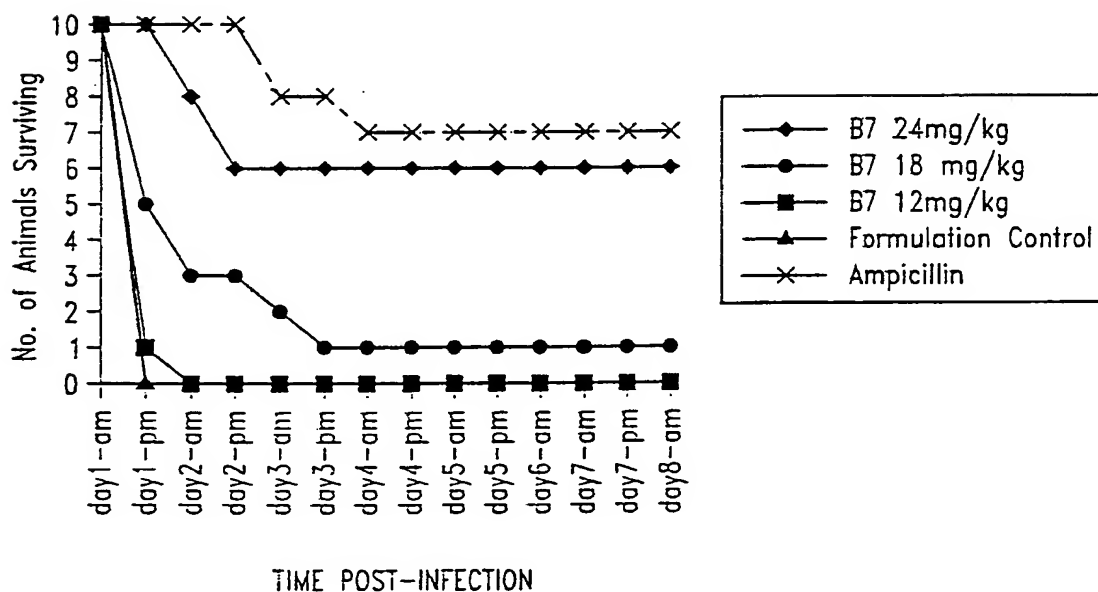
Fig. 17

13/24

Fig. 18
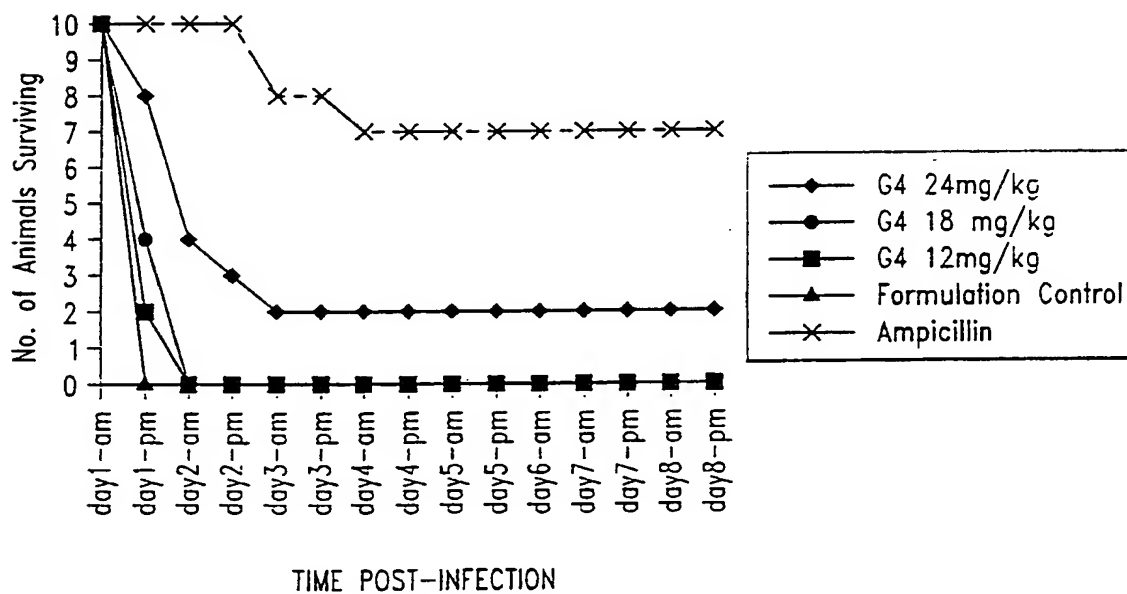
Fig. 19

14/24
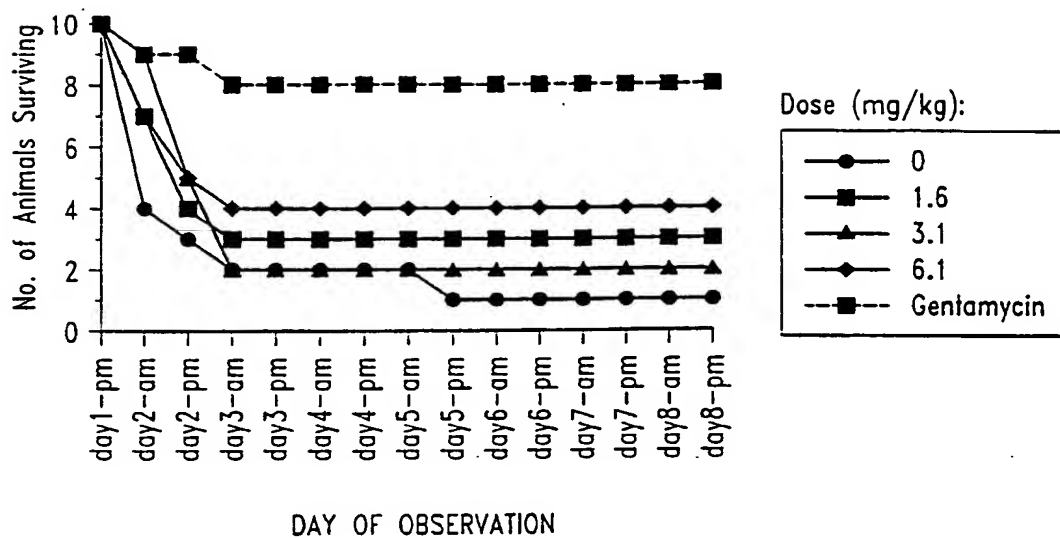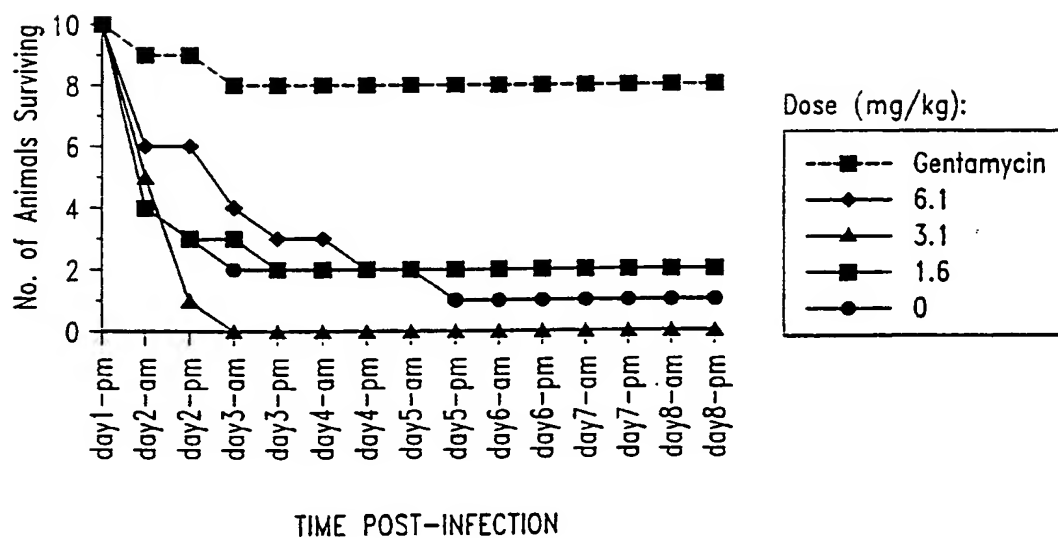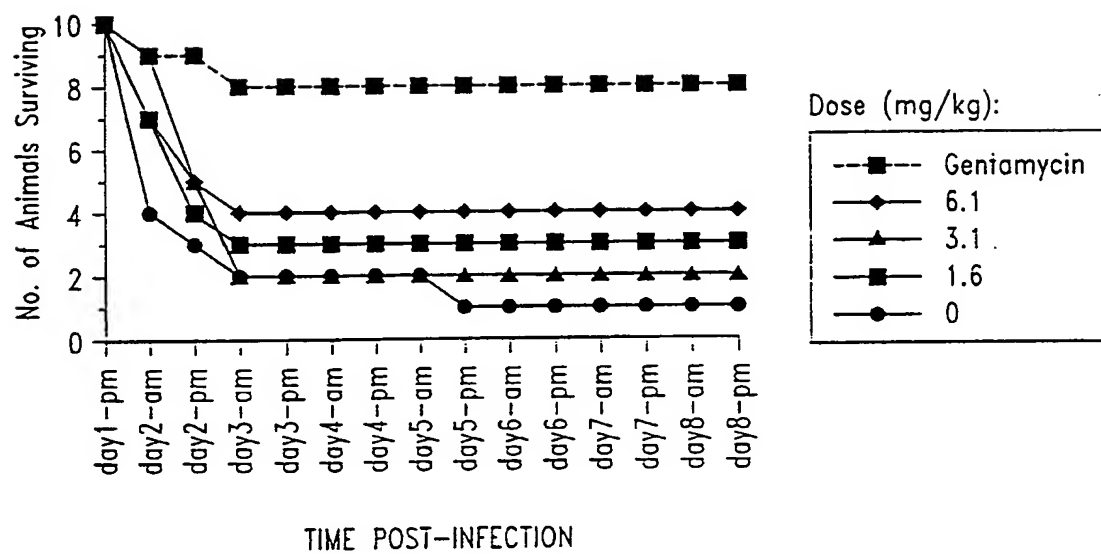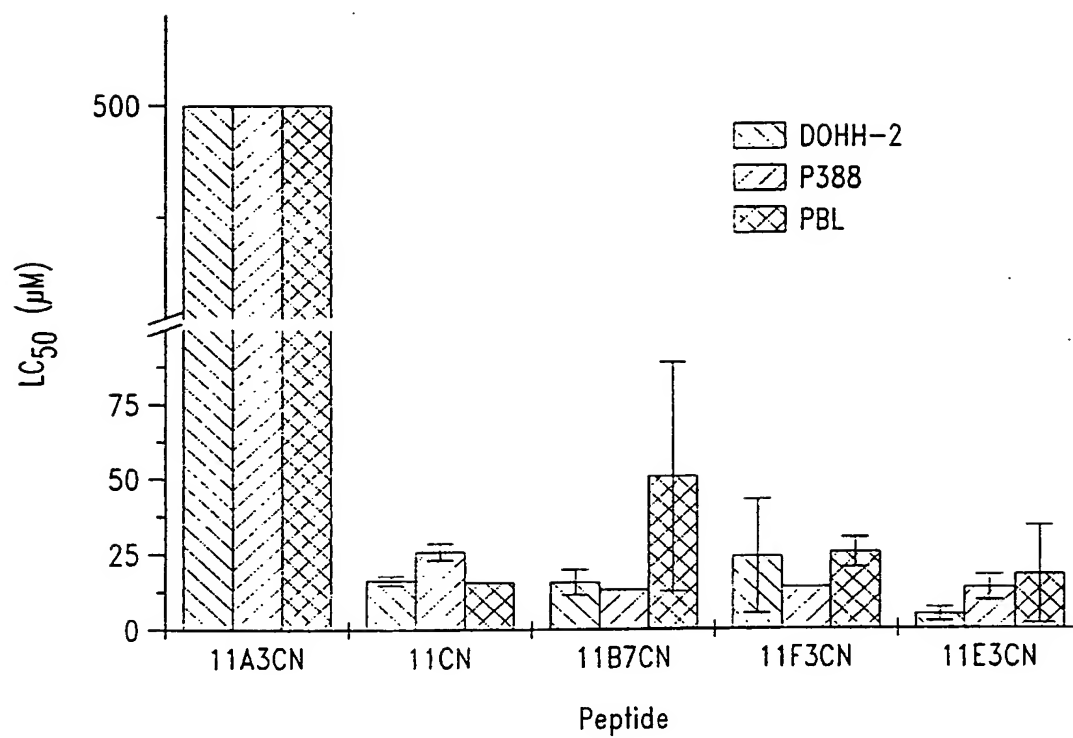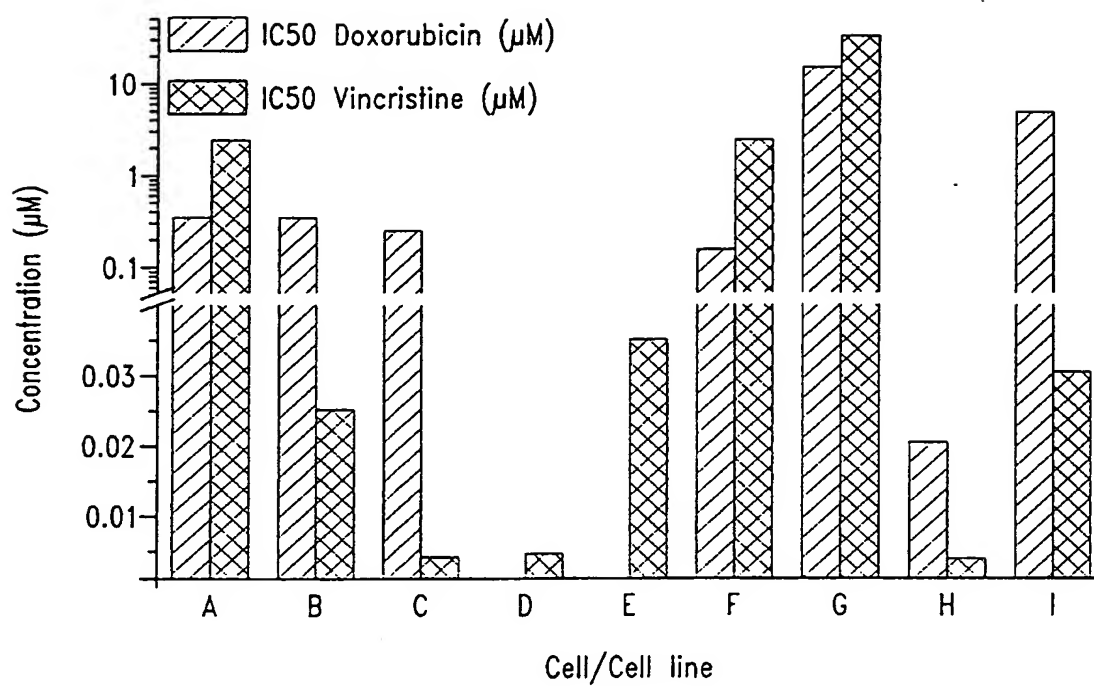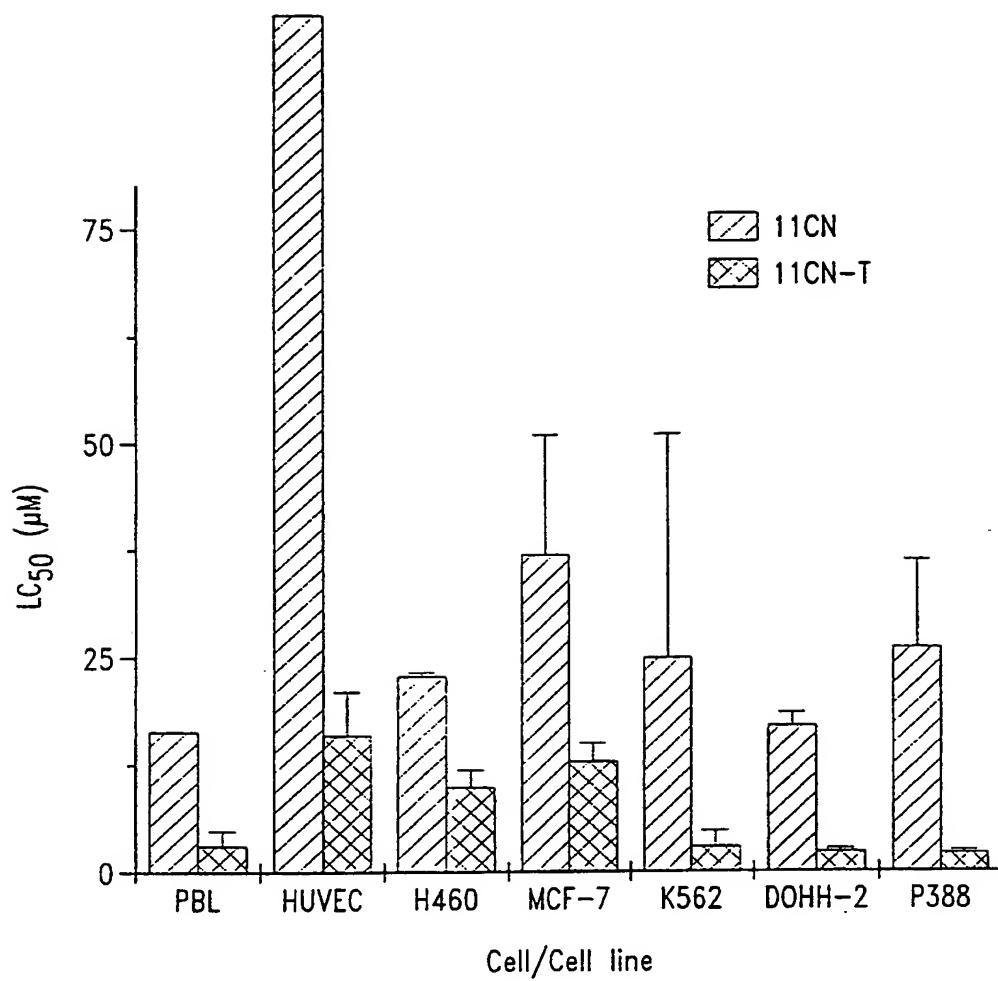
DAY OF OBSERVATION

Fig. 20A

TIME POST-INFECTION

Fig. 20B

Fig. 21

Fig. 22

Fig. 23

Fig. 24
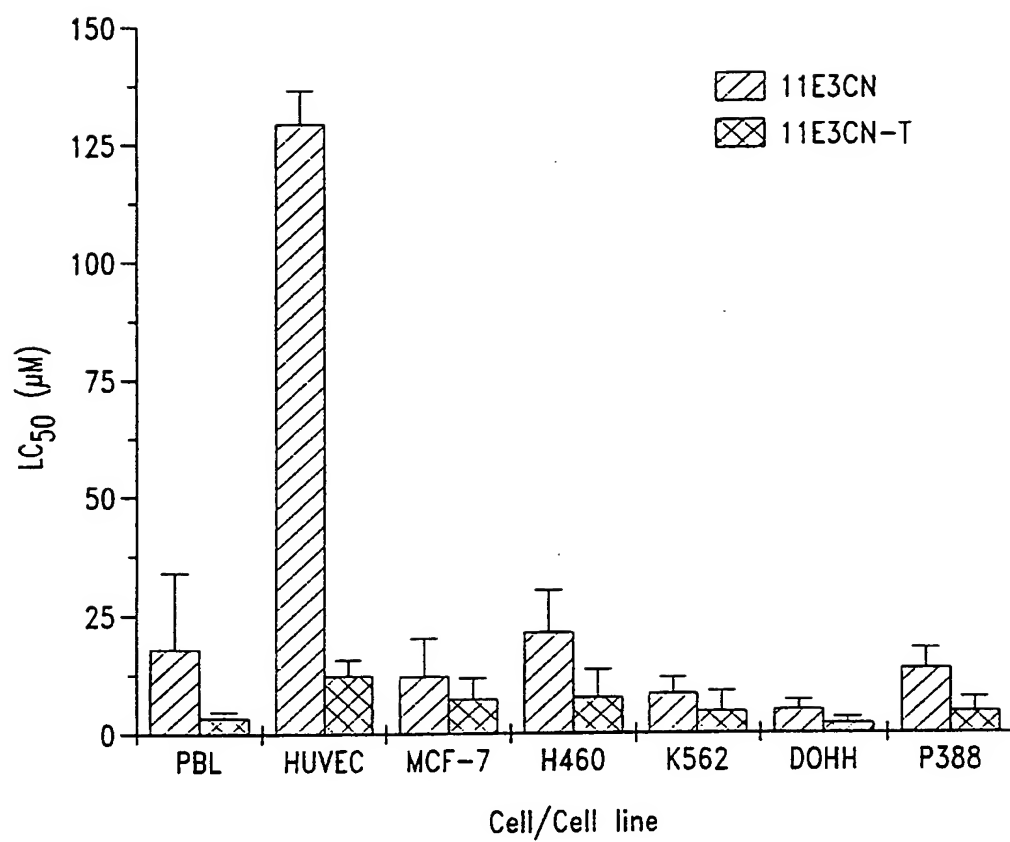
Fig. 25
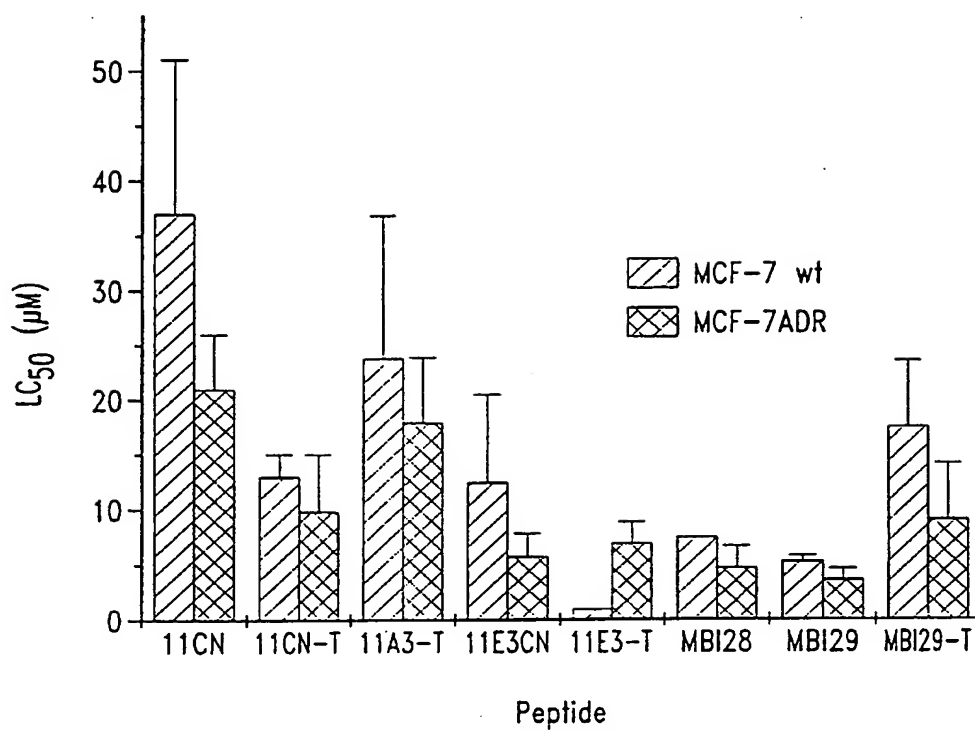
Fig. 26
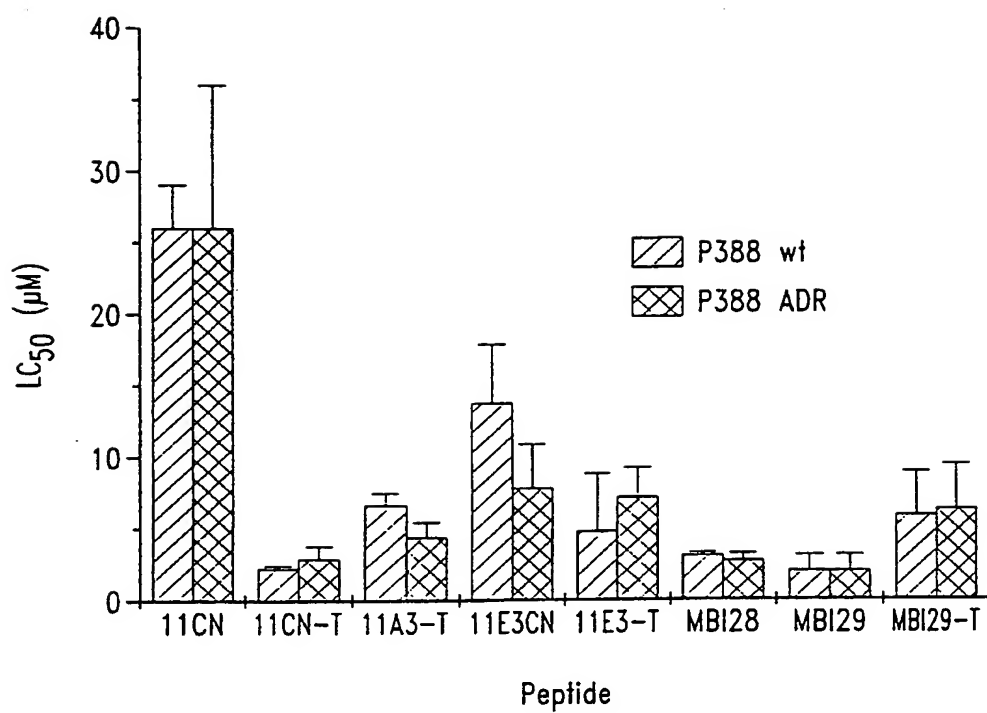
Fig. 27
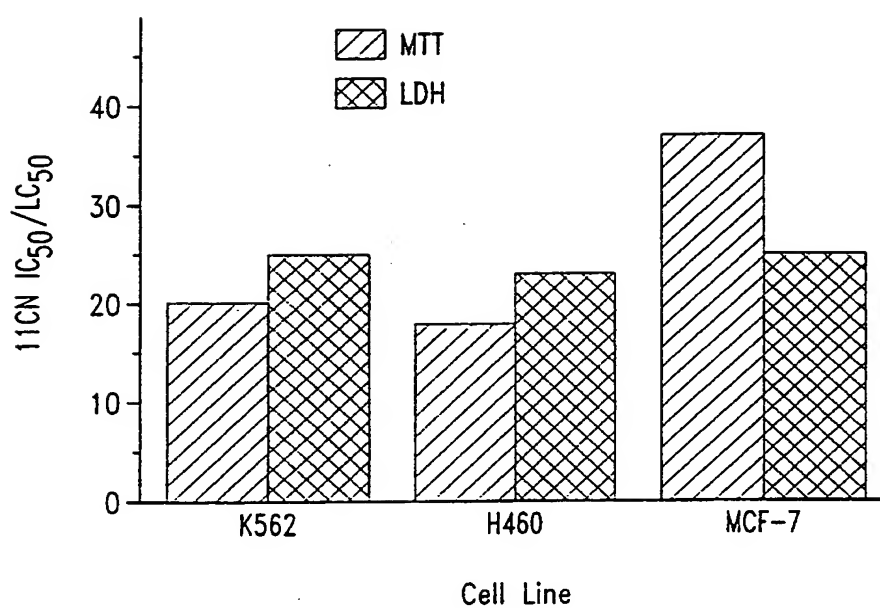
Fig. 28
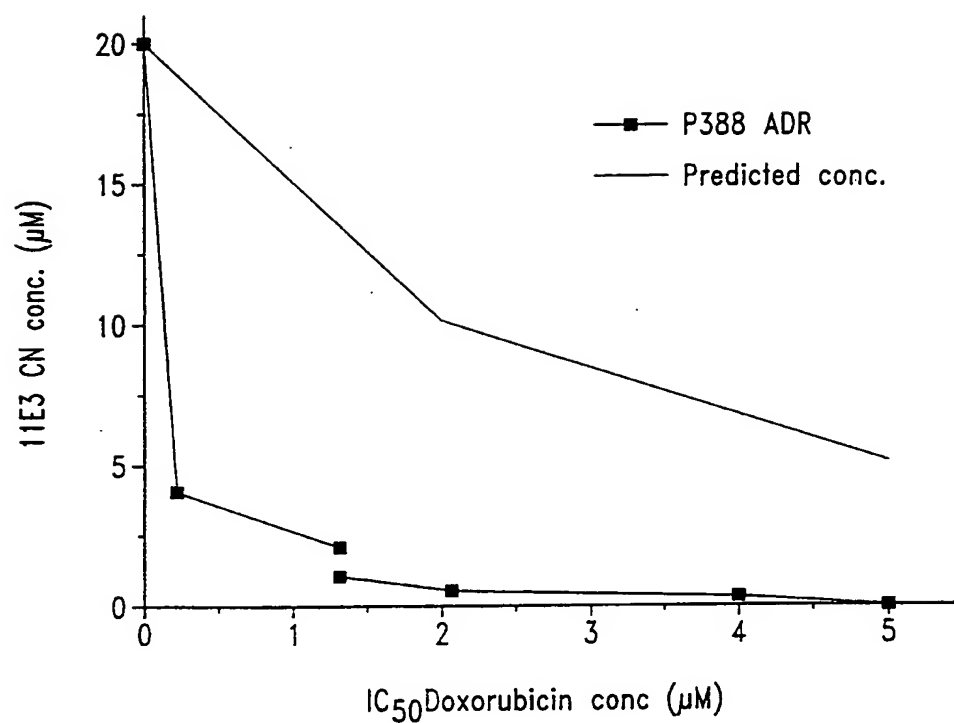
Fig. 29
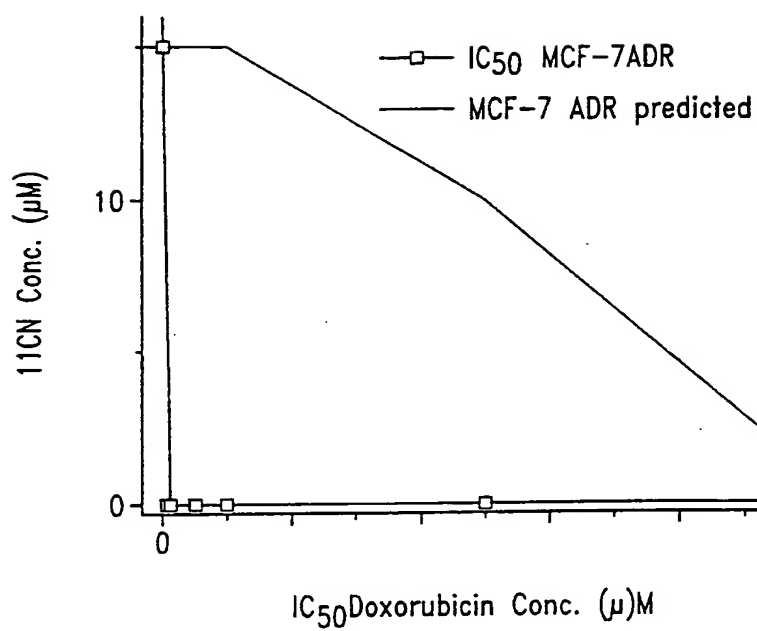
Fig. 30
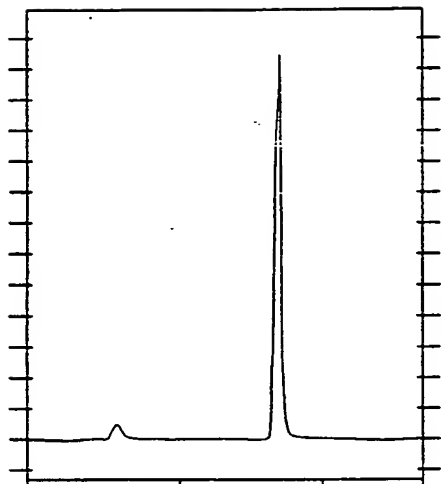
Fig. 1A

Fig. 1B

Fig. 1C

2/24

Fig. 2A